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MICROBIOLOGY

BY

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First Edition

McGRAW-HILL BOOK COMPANY, Inc.

NEW YORK: 370 SEVENTH AVENUE

LONDON: 6 & 8 BOUVERIE ST., E. C. 4

1929

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PRINTED IN THE UNITED STATES OF AMERICA

THE MAPLE PRESS COMPANY, YORK, PA.

PREFACE

The author offers no apology for attempting to discuss molds, yeasts, and bacteria as a group. The many common physiological characteristics of the colorless plant give such a group a unity in spite of the diverse morphology and distant relationship of its components.

He must, however, ask for leniency in critics of the manner in which the field has been covered. One man cannot possibly read the source literature for such a series of organisms with all the ramifications and implications into which it may lead; this must be evident to anyone who knows anything of the mass of data in microbiology that has piled up since 1870. One topic (bios) had about 150 titles listed in 1925; another (bacteriophage) had nearly one thousand, all since 1917. The articles on enzymes are almost like the sands of the sea, and the same is true of those on some of the pathogenic bacteria, such as typhoid and tuberculosis.

The general scheme of presentation has been to describe "types" in considerable detail; a method borrowed from the zoologists. Types have been chosen which are common and have biological or practical importance, if possible, both. As much as possible of both morphology and physiology has been connected to some type of mold, yeast, bacterium, or enzyme and, other facts described in terms of the types.

At times, the type method has fallen down; at other times, common methods and results could be better presented in general chapters. These general chapters are to be found at the beginning and at the end, the bulk of the book being a description of typical species. The beginning (general) chapters are the author's solution of textbook work while a class is learning some necessary elementary technic in the laboratory. The final chapters are an attempt to place before both teacher and student some of the theories, problems, difficulties, and controversies that confront the modern microbiologist. It has seemed well for the student to leave the subject not with the smug complacency that it is all neat, pat, and settled, but rather that he is looking out over a sea of boiling controversy and that many theories that he has been using may be discarded in another generation.

A few of the older pieces of research are given sometimes in considerable detail, many of the later ones are necessarily omitted. Many excellent papers may have been missed in spite of extensive reading during some twenty years of teaching microbiology in various phases to classes. The author would be glad to have any errors of omission or commission called to his attention.

The author wishes to extend thanks to the following colleagues and publishers for literature, photographs, and permission to use illustrations and extracts:

For photographs: To Profs. S. G. Lehman, Hans Molisch, D. H. Jones, E. B. Fred, Philip Hadley, M. H. Soule, and Dr. C. W. Stiles; to Prof. A. Franklin Shull for three illustrations from his "Principles of Animal Biology," to Profs. Eames and McDaniels for the use of the Nägeli's portrait, to Prof. E. B. Fred for the nodule illustration from Löhnis and Fred's "Agricultural Bacteriology." To G. P. Putnam's Sons for the picture of Metchinkoff; to John Wiley & Sons, Inc., for permission to use two figures from Zsigmondy's "Colloids and the Ultramicroscope" (Eng. Transl.); to The Macmillan Company for the use of the map of Plymouth, Pennsylvania, from Frost and McCampbell's "General Bacteriology;" to Williams and Company for three figures from Calmette's "Tuberculosis;" to Carl Zeiss, Inc., of New York, for the loan of electrotypes of lenses, microscope, dark-field condensers, and microdissecting outfit; to the Fleischmann Yeast Company (research division) for material on compressed yeast; to the Magic Yeast Company for material on methods of dry-yeast manufacture; to the Spencer Lens Co. for the loan of electrotype of various types of condensers; and to the Doubleday, Doran & Co., Inc., for permission to use excerpts from their translation of Vallery-Radot's "Life of Pasteur."

He wishes to express his appreciation for the kindness of a number of colleagues in reading portions of the manuscript. They have assumed no responsibility for the manner of presentation, for the ideas expressed, or the content, but they have given valuable assistance and counsel on the topics which they read:

Dr. W. G. Marquette, microscope and ultramicroscope; Mr. A. Gershoy, carbon cycle; Prof. E. A. Van Alstine, nitrogen cycle; Dr. Chas. Thom, molds and microorganisms in foods and canned goods; Prof. F. W. Tanner, yeasts and bios; Prof. M. H. Soule, *Bacillus subtilis;* Prof. E. H. Buttles, *B. coli* and *Mycob. tuberculosis;* Prof. G. H. Burrows, enzymes; Dr. Alfred Larson, *Bacillus anthracis, B. typhosus,* and *B. diphtheriæ;* Dr. Archie Robertson, *Staphylococcus aurantiacus, Streptococcus lactis,* the thermophilic organisms, and the bacteria in milk; Prof. I. C. Hall, *Bacillus amylobacter;* Mr. N. R. Smith, bacteria of the soil, and bacterial life cycles; Dr. C. P. Moat, bacteria in water and ice; Prof. L. F. Rettger, disinfectants and theory of disinfectants; and Prof. Philip Hadley, mutations and Twort-d'Herelle phenomenon.

B. F. Lutman.

Burlington, Vt.
July, 1929.

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MICROBIOLOGY

CHAPTER I

THE CHEMICAL ACTIVITIES OF MICROORGANISMS AND THEIR RELATION TO MANKIND

Microbiology deals largely with the destruction of the organic chemical compounds of plants and animals and with the organisms which cause these changes. Living microorganisms break the complex organic molecules into water and the simplest gases, the process being known as "fermentation" or "putrefaction."

Fermentation of Sugars.—One of the most familiar of these fermentations is that induced in sugar solutions by yeasts; the reaction:

dextrose = alcohol and carbon dioxide

has been so much studied that it may be regarded as the prototype of all fermentations.[5] Sugars are readily destroyed and the yeast plants begin a cycle of changes which converts them into alcohol. The alcohol is attacked by bacteria to form acetic acid, and vinegar eels may then reduce the acetic acid to carbon dioxide and water. The energy stored in the sugar is released in the growth of the microorganisms to appear as heat.

The sugar molecule is valuable to these microorganisms, not alone for the carbon which it supplies in a combined condition, but more particularly for the energy which they derive from its destruction.

Putrefaction of Protein.—In a similar manner, the complex protein molecules in the body of a plant or animal, sometimes containing 10,000 atoms, may be converted finally into water and simple gases such as ammonia, hydrogen, sulphur dioxide, hydrogen sulphide, and methane, with a similar release of energy.

Oxidation of Sulphur, Ammonia, and Other Chemical Compounds. The life processes of the carbohydrate and protein destroyers are carried on by complex reactions, but some microorganisms, however, are able to oxidize simple chemicals such as sulphur, ammonia, hydrogen sulphide, or methane, and from the reaction derive energy for growth and life activities. The carbon which is built into their bodies must come from some other source; the chemical oxidation supplies only energy.

Common Physiological Characteristics.—No morphological characters are common to these microorganisms as they belong to widely sepa-

rated groups of the plant and animal kingdoms. In their physiology, however, two traits are common: (1) a *lack of chlorophyl* and (2) *the need for a food which contains stored energy* which can be released by simplifications or oxidations.

Principally Chemical Problems.—The morphology and classification of the lower types of life had been in the hands of botanists and naturalists during the early part of the nineteenth century, but it was not until the profound change which microorganisms might induce was discovered that they had attention focussed on them. The problems associated with microorganisms are, in general, so chemical in their nature that much of the early knowledge of the fermentative and putrefactive changes and, incidentally, something of the organisms inducing them is due to two great chemists, Justus von Liebig and Louis Pasteur.

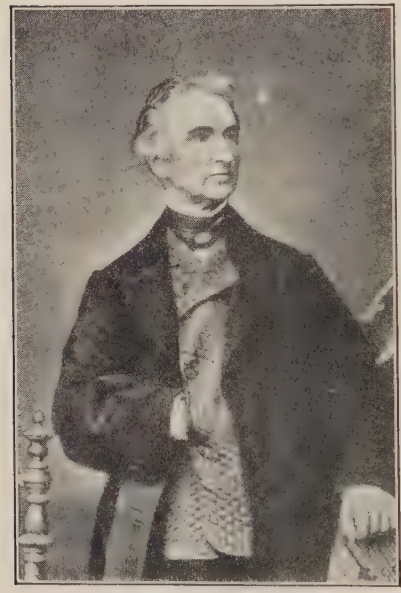

FIG. 1.—Justus von Liebig (1803–1873). Organic and agricultural chemist; pioneer in the world of thought; he, by his chemical training, helped to direct a great epoch of knowledge, the rise of biology.

The Chemical Explanation of Fermentation.—Liebig (Fig. 1), the great organic and agricultural chemist of 1840 to 1870, was very certain that all changes in nature are purely chemical.[2] The organic molecules are so large and complex that they may be easily shaken apart into simpler constituents. Decaying or putrefying material, brought into contact with organic material which gave no evidence of such a breakdown, would communicate its condition and bring out a similar decomposition. Liebig[3] explained not only decomposition but also *disease* on the theory of the *lability of organic matter* in contact with other putrefaction:

"It has been clearly proved that muscle, urine, cheese, cerebral substance, and other matters, in a state of putrefaction, communicate their own state of decomposition to substances much less prone to change of composition than the blood. When placed in contact with a solution of sugar, they cause its putrefaction, or the transposition of its elements into carbonic acid and alcohol. When putrefying muscles or pus is placed upon a fresh wound, it occasions disease and death. It is obvious that these substances communicate their own state of putrefaction to the sound blood *from which they were produced*, exactly in the same manner as gluten in a state of decay or putrefaction causes a similar transformation in a solution of sugar."

The Biological Explanation of Fermentation.—This purely chemical explanation seems to have been unchallenged for many years, when another chemist, Louis Pasteur (Fig. 2), discovered with the microscope that in such changes small organized bodies were always present but seemed to be absent if no change were going on; they produced the change and were "living ferments." The years, 1850 to 1864, were filled with bitter controversy between these two chemists, but Pasteur won in the latter year. The fundamental contributions which he made during these years are, in the main, the bases of modern microbiology, for science is built up from the facts learned in such controversies.

Pasteur was not entirely correct, however, and the problem has moved back into a region, which lies between the visible microscopic forms, which he saw, and the invisible organic agents, known as *enzymes*. These are not so simple, in all probability, as a protein molecule but invisible under the microscope and much smaller than the microorganisms from which they arise. They offer a fascinating field which lies between the sciences of microbiology and chemistry. In their susceptibility to heat and to coagulation they are like all other living organisms but in their activity they are more nearly chemical. They are the "unorganized ferments" of the early Pasteur period, 1860 to 1875.

FIG. 2.—Louis Pasteur (1822–1895). French chemist and microbiologist. Famous for his work on the spontaneous origin of life, on the production of disease by bacteria, and for his cure for rabies.

Specialized Microbiology.—Since microorganisms are so important in the destruction of organic materials, their study has become intensified and specialized. To the public, bacteriology deals only with disease producing forms but such an idea is very erroneous, as the disease producing organisms are the exceptional ones; soil, water, and food are filled with myriads of harmless species, which bring about marked changes in the substances upon which they are growing.

Soil Organisms.—A majority of the organisms are found at one time or another in the soil; in it they pass some phases of their life history and many of them change it chemically. The reactions which they induce in the soil are today considered the most important that go on in this underground laboratory. Liebig believed that he could analyze the soil, analyze the plants which grew on it, and tell the grower of a crop the proper proportions of the necessary constituent chemical elements to add to the soil to grow a maximum crop. We know today that such chemical analyses are practically valueless so far as determining the crop-producing ability of a soil is concerned, while we can learn much about the productivity of soil, or the availability of

substances in a soil, by its use in solutions with microorganisms. We owe much to the early chemists of soil and plant life, but they tried to exceed the capabilities of their science when they tried to extend it to include all fertility problems. The chemical substances may be present in the soil but not in forms that a crop plant can utilize them; they may not be *available*. The availability comes from the activities of these simpler forms of life.

Organisms in Foods.—Food substances fall a ready prey to the microorganisms; and all food preservation, whether drying, canning, preserving, or pickling, is an endeavor to prevent their growth and is based on bacteriological principles. One of the most perishable foods is milk. Milk is a food substance containing many kinds of microorganisms, due in part to its origin and in part to the fact that it is, in itself, an almost ideal substance in which they can grow. The commonest types of milk bacteria attack the lactose of the milk and convert it into lactic acid, but others destroy, or partly destroy, the proteins and render them more soluble, while still others will, in the course of time, break up the fats. Some of these bacteria give flavor and variety to milk products, so that various types of cheese and the ordinary butter are made from cream that has been ripened, *i.e.*, has been soured by these lactic acid-forming organisms. The dairy and creamery industry is based on the control of the microorganisms which occur in the milk and its products.

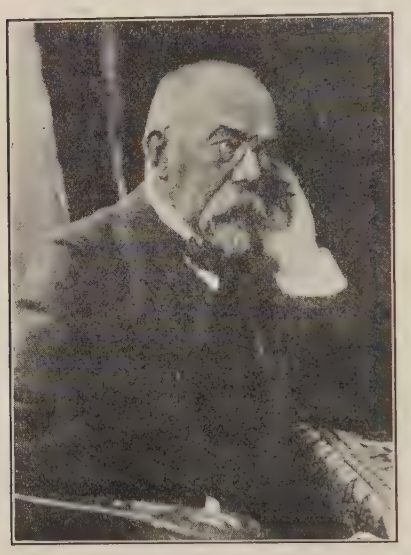

Fig. 3.—Robert Koch (1843–1910). Founder of the germ theory of disease and famous for his work on tuberculosis, cholera, typhoid, leprosy, and tropical diseases.

Organisms in the Food in the Digestive Tract.—We ingest along with the food large numbers of all sorts of microorganisms which continue to change many of the foods. The bacteria are very abundant in the human intestine, as in the intestines of all warm-blooded animals, and play an important rôle in rendering food more soluble, although, at times, they may induce changes that are not for the good of the individual, as their products may be slightly *toxic* or poisonous. Old age and death, according to the theory of Metchnikoff,[4] are due to the accumulation of poisons produced from the destruction of the proteins by the putrefying microorganisms which have been absorbed.

Microorganisms as the Producers of Disease.—The most important bacterial products are those which come from the so-called *pathogenic* or *disease-inducing organisms*. Since the bacteria at times attack us and our domestic animals, they are studied with especial care in order to determine their mode of life, their method of attack, the weak periods in their life cycle, the means by which they get from one host to another, the weapons with which they attack, and the results of their attack. All of these studies we group together under the bacteriology of the pathogenic microorganisms. It forms one of the most important subjects in the medical curriculum today, although 50 years ago the bacteria were disregarded entirely by the medical profession, and these minute forms were turned over to the botanist for investigation. It must be remembered that while it was a German doctor who furnished the decisive proof for the "germ theory of disease" (Robert Koch,[1] 1876,

Fig. 3), he derived his methods and based his work on the labors of a botanist who was interested in bacteria, Ferdinand Cohn, professor of botany in the University of Breslau (Fig. 4). Some very important diseases are the result of the attacks of the lower organisms, and great laboratories and institutes are devoted to their study. One of the most interesting phases of this study of bacteria has been to make serums to neutralize the poisons of the bacteria or to destroy the bacteria themselves. Serums are being used today against almost every bacterial disease. But, more important, epidemics are prevented by isolating individuals who have contracted the disease and who might serve as centers of infection. Modern society lives packed into such crowded spaces that we should have recurring plagues if science by *hygiene* and *preventive medicine* had not made difficult the spread of disease organisms.

The Organisms Involved in These Changes.—The minute dimensions of these organisms put them beyond the unaided human vision. They are microscopic and, hence, have been called *microorganisms* or *microbes* and the subject which discusses them, *microbiology*. Bacteriology implies only a discussion of bacteria, and, while a majority of the pathogenic organisms are bacteria, even medical texts add the yeasts and fungi and some of the protozoa.

Fig. 4.—Ferdinand Cohn (1828–1898). Founder of the modern system of classification of the bacteria.

The organisms which bring about these changes in the organic compounds, reducing them to simpler forms, belong to the colorless plants, the word *colorless* being used only in the sense that they lack the essential chlorophyl characteristic of the common higher plants. We know them as *molds*, *yeasts*, and *bacteria*.*

The molds, yeasts, and bacteria are all exceedingly small, only the molds being visible to the unaided eye. The yeasts and bacteria cannot be seen individually; they can be seen only when heaped together in a "colony." Their size is so small that a special measure, the *micron*, 1/1,000 millimeter = 1/25,000 inch, must be used. The mold spores and the yeast

* The word "microbe" was suggested by Sedillot[6] in a meeting of the Paris Academy of Science in 1878, in the following words: "The names of these organisms are so numerous that they ought to be defined and in part reformed. The word, "microbe" (little life), has the advantage of being short and of a general significance and as my friend M. Littre, the most competent linguist in France, has approved, we should adopt it and discontinue the use of the present names for the designation of these varieties of organisms more particularly studied." Pasteur approved the suggestion and the name has passed into general use especially in France. It may be pointed out, however, that the word is literally incorrect since it means little life, very different thing from little living things.

plants are almost as large as the little particles of dust which float at times in the air, but hundreds of the bacteria of the smaller types, since they are often only 1 or 2 microns in diameter, could find a resting place at one time on these same little motes. The larger ones are only 10 or 20 microns in length, while the smallest are only a fraction of a micron in diameter and lie almost at the limits of the microscope. Some idea of the relative sizes can be derived from Fig. 5.

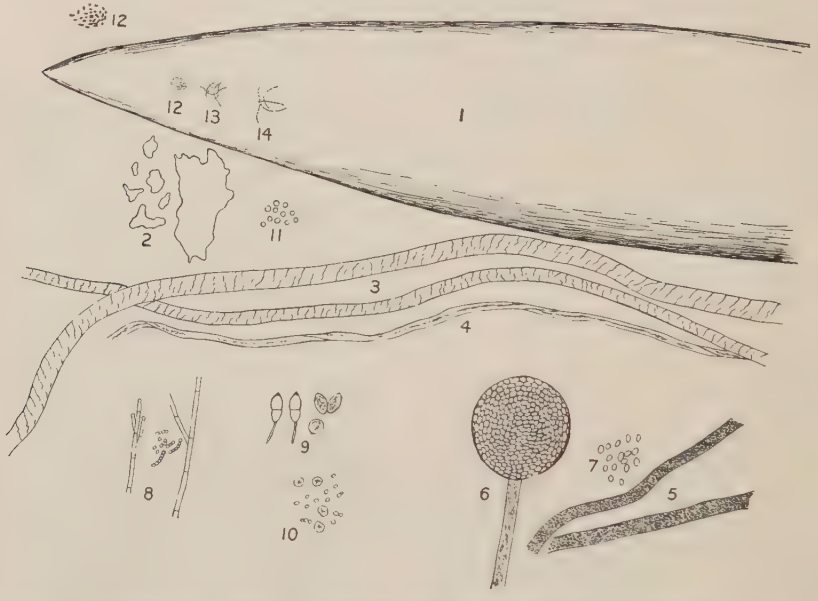

FIG. 5.—Comparative sizes of objects under the microscope; all magnified about 100 diameters: 1, the point of a very fine needle; 2, dust particles caught from the air; 3, wool fibers; 4, cotton fibers; 5, hyphæ of *Rhizopus nigricans;* 6, Sporangium of same; 7, spores; 8, conidiophore and spores of Penicillium; 9, spores of the grain rust, *Puccinia graminis;* 10, yeast cells and starch grains from a cake of yeast; 11, human blood corpuscles; 12, the diplococcus of pneumonia; 13, a streptococcus; 14, anthrax bacilli; 15, wild yeast from the air.

THE POSITION OF THESE ORGANISMS IN THE PLANT KINGDOM

Since the organisms are plants, it may be well to recall that the botanist divides them into the following four large groups: the *Spermatophyta* or seed plants, the *Pteridophyta* or fern plants, the *Bryophyta* or moss plants, and the *Thallophyta* or thallus plants. The latter group contains the representatives which are invisible to the naked eye. The thallus plants are a rather difficult group to define, since they seem to have so little that is common to all of the members included. In general, it can be said, however, that they never develop a true stem, root, or leaf, although they have organs which resemble them and serve the same functions. Since this group contains the organisms considered in such a

study, the following classification may be of help in placing them among the *Thallophytes:*

THALLOPHYTA

1. *The algæ:*
   - *A.* The *Blue-green algæ*, blue-green plants with cyanin as one of the pigments
   - *B.* *The green algæ:*
     - *a.* Protococcales, one-celled algæ
     - *b.* Confervales, composed of many thread-like or membraneous algæ
     - *c.* Siphonales, tube-like forms composed of a single cell
     - *d.* Conjugales, sexual reproduction by conjugation
   - *C.* The *brown algæ*, the kelps, wracks, sea palms
   - *D.* The *red algæ*

2. *The fungi*
   - *A.* *The bacteria*
   - *B.* *The filamentous fungi.*
   - *C.* The *phycomycetes* thread-like molds composed of a single cell
   - *D.* *The ascomycetes*, fungi which reproduce by means of spores in sacs called *asci.*
     - The common green and blue mold
     - The mildews
     - The cup fungi
     - The yeast
   - *E.* The *basidiomycetes*, fungi in which the spores are borne on a basidium
     - *a.* The smuts of grains and grasses
     - *b.* The rusts
     - *c.* The puff balls, shelf-fungi, and mushrooms

The two groups run parallel, as they are probably, in most cases, very closely related. The fact that one series has chlorophyl and the other lacks it is merely incidental to their resemblance in form, reproduction, and structure. The fungi are offshoots from a series of groups of the algæ and one of the problems of the morphologist is to determine those relationships.

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CHAPTER II

THE APPARATUS OF THE MICROBIOLOGIST

The microbiologist is able to see these minute forms only by the help of the compound microscope, but some of them are too small even for its magnifications, and reliance must be placed on the ultramicroscope and filters to determine their presence, size, and form.

THE COMPOUND MICROSCOPE

Since all of these organisms have to be studied by the help of the microscope, the advance of microbiology has been practically a history of the microscope. Whether the Dutch lens grinder, Jannsen, discovered the principle of the compound microscope or whether it was an Italian invention, the improvements which were made about the beginning of the seventeenth century were by a number of workers. Simple lenses and their properties had been known to the Romans, but the compound microscope with its magnification of 50 to 3,000 diameters opened a new world which amateur lens makers explored, as amateurs today spend their time with radio or electrical contrivances. A training in lens grinding preceded any examination of this new and fascinating region, but some amateurs became so skillful in grinding lenses accurately that they produced microscopes with a magnification of 300 or 400 diameters. The high-power lens on the ordinary student instrument of today gives a magnification of 400 to 500 diameters, so that these homemade instruments were real compound microscopes. They were ground, however, to no fixed formula as to the shape and glass. A modern lens in not an instrument that is made and improved by progressive trials, but is ground to an exact curvature from glass with known exact refractive index and dispersion. With these primitive instruments, however, one enthusiastic amateur was able to see bacteria. Leeuwenhoek, a Delft Dutchman (1632 to 1723), (Fig. 6) drew them with an accuracy and skill that would be a credit to a student equipped with a much better outfit. On Sept. 14, 1683, he communicated his discovery in a letter to the Royal Society of London.

It is interesting to note that, although Leeuwenhoek discovered bacteria in 1683, Linnæus, the great Swedish botanist, when writing a systematic account of the plant kingdom, named and classified a few of the larger fleshly fungi and then grouped all the other small, unknown, and undescribed plants into the great order of "Chaos."

Oil Immersion Objectives.—The bacteria continued to be studied by such lenses as were available during the eighteenth and nineteenth centuries, and the botanists who specialized in bacteria laid the foundations for our modern classification. About 50 years ago (1879) the microscope was further improved by the homogeneous oil immersion objectives, and magnifications up to 1,000 diameters could be obtained. The very smallest living organisms could be studied with greater ease when they were placed under such relatively high powers. This principle was not a new one, for water had been used as a substitute for the air intervening

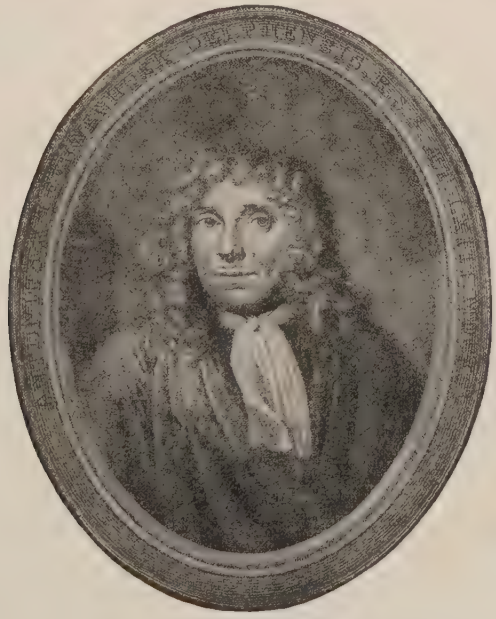


FIG. 6.—Antony van Leeuwenhoek (1632–1723). Microscopist and discoverer of the bacteria (1687). (*From "Garrison's History of Medicine."*)

between objective and cover glass by Amici in 1840 and Hartnack in 1855, glycerin by Gundlach in 1867, and various oils by Amici in 1869. The new principle was to use an oil which had approximately the same refractive index as glass, cedar oil most nearly answering this requirement.

Apochromatic Objectives.—In 1886, a still further improvement in the lenses was made by the invention of *apochromatic* objectives with compensating oculars. These apochromatic oil immersion objectives, made up of at least eight different lenses, were so perfect that a magnification of about 3,000 diameters was possible. This improvement was due to Ernst Abbe, a physics professor in the University of Jena (Fig. 7), so whatever laurels bacteriology and cytology have gathered in the past 50 years, a large part of them must be laid at the feet of the man who has

given microbiology the tools with which to work. Today, the bacteria and even many details inside them, in spite of their almost incredible smallness, can be seen. This advance was made possible by the invention of the new types of glass which had different refractive indexes and dispersions from the old crown and flint glasses which were all that lens makers had formerly at their disposal. Otto Schott, a practical glass maker and chemist, found that, by adding the proper chemicals, baryta, borate, phosphate, and zinc, glasses could be produced which had very different properties from the flint and crown glasses. By combining some of these glasses, Abbe was able to build up a lens which produced an image that was not only color-free, *achromatic* (a = without; chromatic = color) but which had extraordinary definition and freedom from distortion.

Fig. 7.—Ernst Abbe (1840–1905). Professor of physics at the University of Jena; originator (1886) of the apochromatic objective.

These high-power objectives are smaller than the head of an ordinary brass pin so that they could not have been used, if, at the same time, some system of concentrating the light had not been invented. We owe to Abbe the illuminating apparatus or condenser which furnishes better illumination and a better microscopic image. This was invented in 1872.

The Ultra-violet Light Illumination. The physicists have helped biology by producing the wonderful instruments by which so much has been seen, but they can help further by producing a microscope which will make visible still more minute objects.

The present microscope cannot be further improved, since the wave length of light is not much shorter than that of the objects examined. When the objects are so small that they approximate a wave length, diffraction and mutual interference appear and the image is blurred, but, the shorter the wave length, the smaller the objects which can be seen distinctly. In the spectrum, the short waves are at the violet end; beyond the violet is the ultra-violet. By using light with extremely short wave lengths, objects too small to be seen by ordinary light have been photographed. The ultra-violet light which is used, however, unfortunately cannot be seen by eyes not attuned to waves as short as those used for this work. The photographic plate can see what the human eye cannot (except on special screens) but the photographic plate records the objects and it can then be examined. The aid of ultra-violet light will probably not add more than a few thousand diameters to the

magnification, but images of small objects come out much sharper than with lights of longer wave lengths, such as those in daylight.

Barnard[1] claims that ultra-violet illumination and photography represent the only real advance in microscopy in the last 30 years, since the limits of visibility of objects are dependent not only on the size of an object but also on the difference in refractive index which it has, as compared with the medium in which it lies, and on the intensity and color of the illumination.[11]

In order to use ultra-violet light, microscope lenses must be made of a substance through which the rays will pass, as ordinary glass filters most of them out. Koehler of Jena devised quartz objectives in 1904, and these objectives, using glycerine instead of oil as an immersion medium, have been in use in America and Europe since about that date. The cover glasses and slides must also be made of quartz. The light is produced by an electric spark between two electrodes of magnesium or cadmium. Such an instrument cannot be used for examination with the human eye (except by the use of a special screen), since these rays are invisible. Ordinary light can be used for searching for objects; the ultra-violet light can then be turned on and the focus changed a predetermined amount, depending on the wave length of the light used. The record must then be taken on a photographic plate.

J. E. Barnard[1] photographed organisms from the filtered extract from a mammalian cancer. The nature of these bodies from the tumors is as yet undetermined but they seem to be in the filtered extract, and to be photographable.

THE ULTRAMICROSCOPE

The world is seeking today an instrument that will enlarge objects so that the molecules and atoms can be seen. An ultramicroscope,

FIG. 8.—Richard Zsigmondi, joint originator with Siedentopf of the ultramicroscope which is also shown.

first described by Siedentopf, having been devised by him with the cooperation of Zsigmondy in 1903 (Fig. 8), immediately aroused great

interest in all departments of science. The principle involved is that of an intense illumination of particles held in suspension in a liquid, the light coming *from the side and above the objects* instead of from *below and through the objects.* In order to heighten the contrast, the background is as dark as possible so that the method is sometimes known as *dark-field microscopy.*

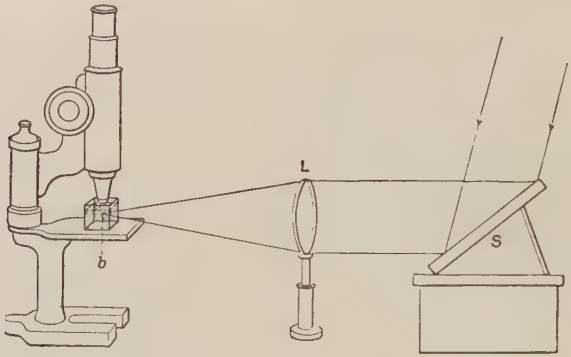


FIG. 9.—Diagram to show principle of dark-field microscopy. (*From Zsigmondy's "Colloids;" John Wiley & Sons, Inc., N. Y., Pub. Eng. Trans. "Colloids and Ultramicroscopy.*)

The effectiveness of the ultramicroscope is dependent upon the illumination by a very intense surface light of only a few particles in the field, the illumination being controlled so that none of it falls on objects above or below the plane that is being examined.[1] Motes, brilliantly

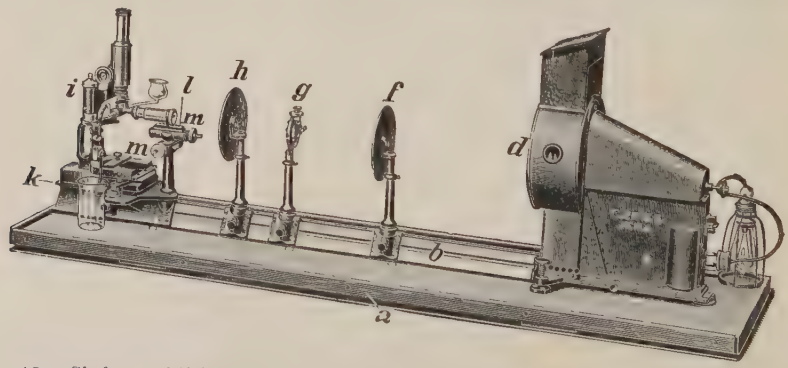


FIG. 10.—Siedentopf-Zsigmondy slit ultramicroscope; about one-eighth actual size. (*From the Carl Zeiss Company.*)

illuminated, dance and shine in a ray of sunlight which falls into a darkened room, while we should be absolutely at a loss to find or to see the same sized particles by looking for them against a background of almost their same color. The background must be dark and the particles must be brilliantly illuminated. The principle upon which it depends is shown in a simple form in Fig. 9, one of the latest models in Fig. 10.

Objects to be examined must be suspended in a liquid which is brilliantly illuminated by a beam of light from above and to one side; the best light being either from the sun or an arc. This ultramicroscope will make particles visible that are much less than $\frac{1}{4}\mu$ in diameter, this size being the limit of the ordinary microscope (Figs. 11 and 12). The

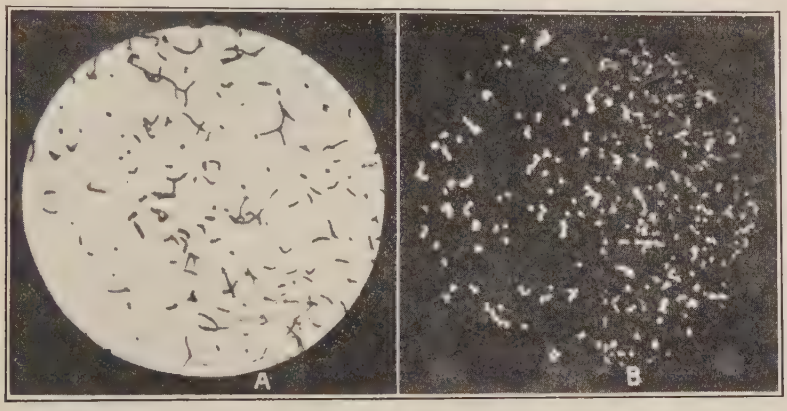


FIG. 11.—The same organism, *B. typhi-antityphosum*, with bright- and dark-field illumination. (*From Almquist.*)

instrument is not perfect; we can see only the *relative size of objects*. The outlines are not distinct but by means of them much has been learned about solutions and colloidal gels. The unit of measurement with the ultramicroscope is a micro- μ ($\mu\mu$) = 1/1,000,000 millimeter. Since the limit of visibility is $\frac{1}{4}\mu$ or $250\mu\mu$ for the best lenses of the ordinary instru-

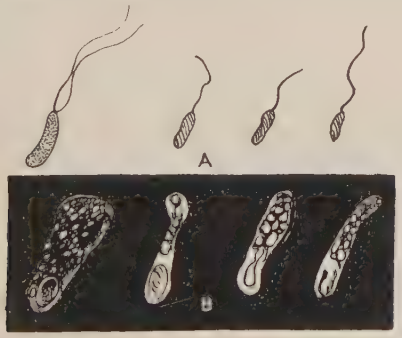


FIG. 12.—*Microspira comma*, usual and dark-field illumination. (*After Gaidukov.*)

ment, these smaller particles are called *ultramicroscopic* or *submicroscopic*. The limits of the ultramicroscope with good illumination are particles of a length of $15\mu\mu$ although, with brilliant sunshine on a clear day, particles almost as small as $5\mu\mu$ can be seen. The relative sizes of some of the objects which have been examined are shown in Fig. 13. The instrument

was originally designed for obtaining some idea of the relative size of colloidal gold particles and many of the figures refer to them.

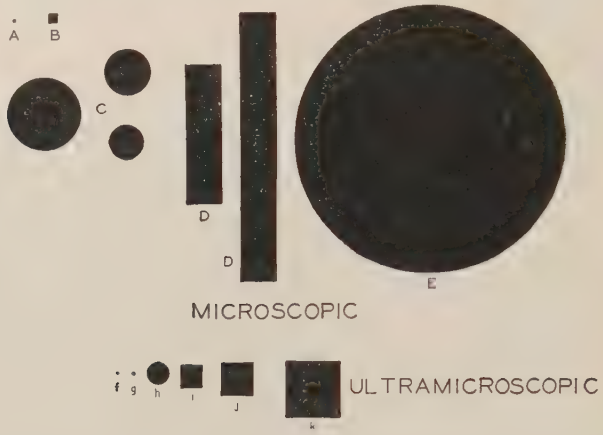


Fig. 13.—Relative sizes of particles seen under the ordinary microscope and under the ultramicroscope: *A*, colloidal gold suspension particles (0.006–0.15 micron); *B*, settled gold suspensions, (0.075–0.2 micron); *C*, various types of cocci (0.5–1 micron); *D*, anthrax bacilli (4 × 1 microns, 10 × 1 microns); *E*, human blood corpuscle (7.5 microns); *f*, hydrogen molecule; *g*, alcohol molecule; *h*, molecule of soluble starch; *i*, *j*, and *k*, smallest particles of colloidal gold that are visible under the ultramicroscope. *A-E*, × 10,000 diameters; *f* to *k*, × 1,000,000 diameters. (*After Zsigmondy, John Wiley & Sons, Inc., New York, Pub. Eng. Trans.*, "*Colloids and Ultramicroscopy.*")

The relative sizes of particles visible to the eye or the various lenses and lighting systems[7] are shown in the following tables:

Table 1

Macroscopic 1 centimeter 10^{-1}	Microscopic 10^{-2} 10^{-3} 10^{-4}	Submicroscopic 10^{-5} 10^{-6}	*a*-microscopic 10^{-7} 10^{-8}
Limits of visibility to eye....	Dry lens, immersion, ultra-violet microscope	Ultramicroscopic	

Table 2.—Possibilities of Resolution

Eye	0.07 –0.08*mm*
Microscope (dry lenses)	about 0.5μ
Microscope (immersion lenses)	0.3 –0.4μ
Microscope (ultra-violet light)	0.15 –0.2μ
Ultramicroscopic, gold particles	0.006μ
Ultramicroscopic, organic colloids	0.003–0.004μ
Polarization microscope or X-rays	

Defects of the Ultramicroscope.—Definition has always been the weak point of the dark-field instrument, but this has been somewhat bettered[23] by changing the numerical aperature, by using a mirror for illumination, by placing an iris diaphragm inside the oil immersion lens itself, and by somewhat modifying the ocular. The

instrument still leaves, however, much to be desired, and the original principle is the same.

The distortion of lines and surfaces as seen under the ultramicroscope can be illustrated by Fig. 14.[25] The one-sided lighting of a flat object, such as that in *A* would show it as a faint line without any breadth, but if the object were tilted slightly,

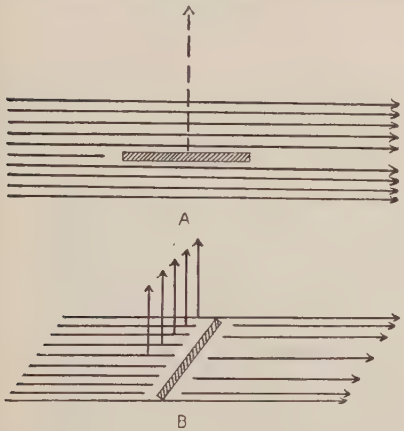


FIG. 14.—The effect of tilting on the apparent size of an object illuminated from side only. In *A*, it appears as an illuminated line; in *B*, the whole plane serves as a reflector. (*After Szegvari.*)

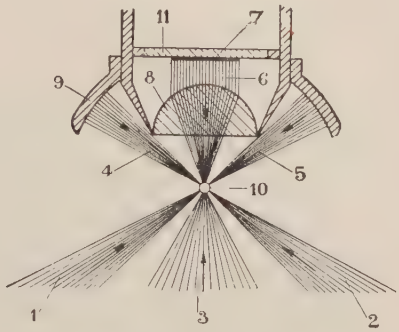


FIG. 15.—Attachment to the objective devised by Spierer; 40 per cent of the light passes through the object as in the ordinary instrument and 60 per cent is reflected on it from above as in the dark-field instrument.

the apparent size would be greatly increased, since more of the surface would act as a mirror or reflecting surface, *B*.

Spierer[23] has suggested an ingenious combination of transmitted combined with reflected light obtained by an attachment to the front of the objective, as shown in Fig. 15. About 40 per cent of the light passes through the object and the other 60

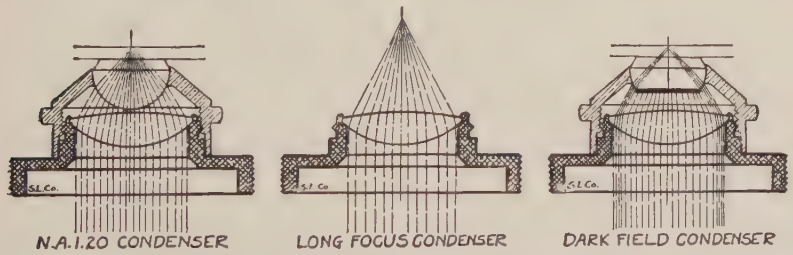


FIG. 16.—Path of the rays of light through an ordinary condenser, a long focus ordinary condenser, and a cardioid condenser. (*Courtesy of Spencer Lens Co.*)

per cent of the light passes around it, to be reflected from the two surfaces at (9), attached to the objective. By the use of this combination of dark-field and transmitted illumination both the internal structure and the surface appearance can be seen through the same lens at the same time.

The Parabolid and Cardioid Condensers.—Results similar to those of the ultramicroscope can be obtained by using a condenser which concentrates the light above the object, as is shown in Fig. 16. This

outfit can be used on any good microscope with high-power lenses and gives very beautiful pictures of small objects, such as bacteria, as seen by light reflected from their bodies with a dark-gray background, against which they appear (Fig. 11). It is in general use for examining bacteria and protozoa in a living condition and for many processes is more useful than the ultramicroscope.

Szegvari[25] has devised and used with the cardioid condenser a shutter with two holes, opposite each other, through which the light may pass to illuminate *the two sides* only of the object (Fig. 17) under observation; this lighting gives a more nearly correct picture than an illumination on all sides or from one side only.

X-rays as a Source of Light.—The molecular structure of organic substances of homogeneous composition such as the cell wall of fibers, can be investigated by the

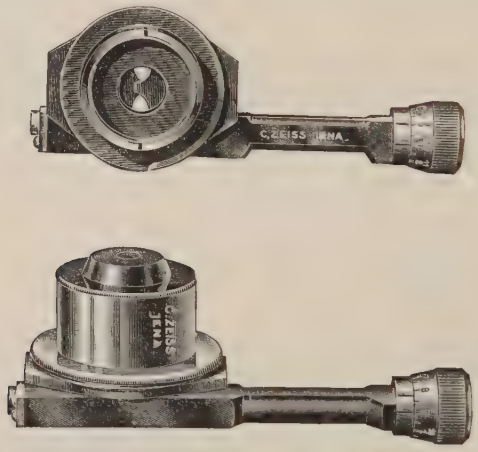


Fig. 17.—Szegvari's azimuth diaphragm condenser, a supplementary attachment to the cardioid condenser for the suppression of undesirable linear details in the dark field image. From above and from side. (*From Carl Zeiss Company.*)

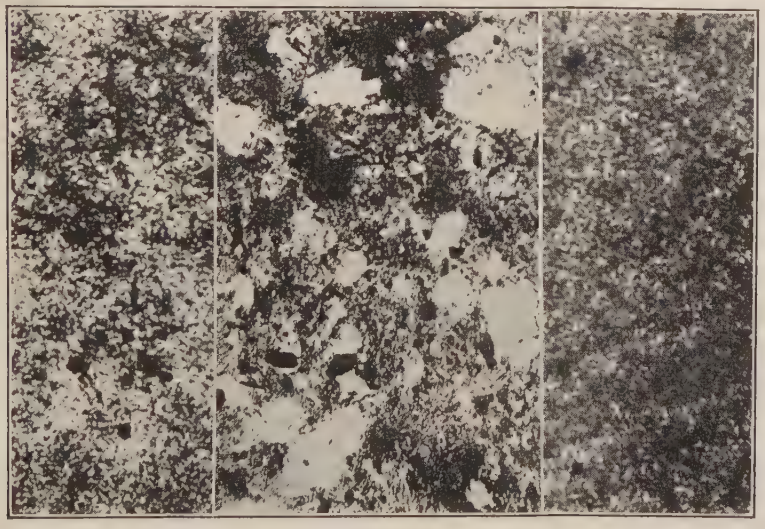
use of X-rays,[24] but the application of this light to colloids of uncertain and varied constitution would not yield as definite results. The same difficulty is met in the use of polarized light, although it, also, can be the illuminant for some similar homogeneous materials.[7]

What should we like to see with the ultramicroscope that we cannot see with ordinary instruments? We should like to see the structure of the colloidal substances of the cells; how the microorganisms break up the higher compounds of carbon; how they throw off the enzymes with which they dissolve their food; how they develop the toxins with which they poison cells; in fact, the whole chemistry of their activities.

While, at the present time the ultramicroscope is, in a way, only a hope, it may be perfected so that we may see down almost to a molecule. In the meantime, microbiologists will work on with the instruments that we have, for there is still much to be learned from their use.

Meanwhile, some more brilliant brain may evolve an instrument along some principle of which we, as yet, know nothing. Science is, after all, only a working and a hoping, and in our stead shall stand some day another generation who shall know all that we know, who shall profit by our mistakes, who shall see farther into the gloom that surrounds us, who shall find paths where we see only bare, trackless cliffs confronting us.

The Use of Filters to Determine the Size of Organisms.—Filtering liquids to free them from bacteria by the use of unglazed clay bougies of candle-like form originated in the Pasteur laboratory and the filters



1 2 3

FIG. 18.—Size of pores in three bacteriological filters. These microphotographs were made of sections of the filters ground thin enough to examine under the microscope. The pores show white. 1, Doulton filter, 2, Berkefeld; 3, Pasteur. × 100. (*From Craw.*)

are known as "Pasteur-Chamberland," although a number of other makes, such as the Berkefeld V, are on the market today. The size of the pores varies, being dependent on the coarseness of the clay particles which compose the wall.

Some of these filters are, at first, very efficient for the removal of bacteria, but later, the liquid passing through the filter walls contains organisms. Three or four days is the longest time that any such filter can be considered safe to remove bacteria. The passage of the liquid through such a filter is by means of very fine, tortuous pores which extend all the way through the clay. The efficiency of a filter is dependent on the size of its pores. Figure 18 shows the comparative size of the pores in the Doulton, Berkefeld, and Pasteur filters. The Doulton and Pasteur filters will remove small organisms, such as those of typhoid,

even at the start of an experiment. The enormous size of the pores in the coarse-grained Berkefeld enables the bacteria to find their way through them immediately, if the liquid is maintained under any pressure. The Doulton is the safest filter;[2] it can be operated for 4 or 5 days and even then only a few organisms pass it. While such filters are useful in tropical countries and on shipboard where water must be rendered sterile for drinking purposes, nearly all types allow the passage of organisms, in time, and must be dried and baked at a very high temperature to clear the pores.

The Berkefeld is now made with a variety of pore sizes, for laboratory purposes. The manufacturers grade these filters according to the rapidity with which fluids pass through them; the BV has a pore size of 0.38μ and the BN of 0.45μ.[15] This sized pore will exclude all organisms except those of the size of *Vibrio percolans* with a diameter of 0.31μ, *B. prodigiosus*, 0.31μ in diameter, or the globoid bodies of poliomyelitis, 0.2μ.[6]

The chemical factory of de Haen of Seelze, near Hanover, manufactures a filter with a maximum sized pore of 1μ in diameter. This filter[21] will securely hold back all except the smallest visible bacteria and, as it has a smooth surface, may be dried and used for dry-weight determinations, something which was impossible with the old, rough, unglazed filters.

The statement that a filter will remove all organisms is, it will be seen from the above facts, only relative, since the bacteria vary so much in size that the filter must be very fine-pored to prevent the passage of all organisms, in fact such an assertion only means that it will remove all the ordinary types over 1 micron in diameter. The bacteria swim through the pores but if their motility is suppressed by cold or narcotics, such as ether or chloroform, their passage is prevented.[15] The investigation of Mudd[16] who used a filterable organism, *Vibrio percolans*, and the very fine-pored Berkefeld V filter where the pores narrow down to 0.4μ in diameter, showed that these organisms had difficulty in swimming through the tortuous channels of the filter toward the cathode. The organisms could find their way with the current toward the electrode much more rapidly than if unaided, the electric current apparently serving as a guide to point out the proper general direction. Or, the filter itself may be electrically charged and remove colloidal particles which have the same charge while those with the opposite charge are retained by the filter.[9]

IS THERE AN ULTRAMICROSCOPIC WORLD?

The very best lenses of the apochromatic type will not distinguish objects less than $\frac{1}{4}\mu$ in size and even with a special immersion lens and violet light, the limit[14] is 0.12μ.

One of the smallest known organisms is the corkscrew-shaped *Spirillum parvum* (Esmarch's organism), which is only $1 \times 0.3\mu$ and so thin

that it will pass through the ordinary Pasteur or Berkefeld filters, but may be seen with the apochromatic oil-immersion lenses.

The introduction of the ultramicroscope gave hope that we should see still smaller organisms than the bacteria swimming around among the comparatively large species. Gaidukov[8] found forms which looked very much like the bacteria; some were rods, others, corkscrew-shaped, but among these motile forms were others which were non-motile and may not have been bacteria. Raehlmann[17] found similar bodies in decaying protein solutions.

On the other hand, Molisch,[14] after numerous observations of all sorts of material under the microscope and the ultramicroscope, came to the conclusion that there was nothing that could not have been seen with the ordinary instrument by the use of the best illumination. He further strengthened his observations with the microscope by the fact that he had never been able to isolate, on plates, pure cultures of any bacteria which he could not see under the ordinary microscope. They certainly should be found occasionally on ordinary culture plates if they are as abundant in nature as Gaidukov believed.

Miehe[13] has tried to answer the question as to the existence of ultramicroscopic forms by filtering out (through a de Haen filter) all visible organisms from various liquids. The supernatent water from soil, aquarium water, sewage, milk, hay infusion, and impure cultures of Azotobacter and Amylobacter were treated in this way and then placed under the ultramicroscope after having been given a preliminary examination under the ordinary microscope. The filters were of such porosity that they passed some of the smaller bacteria, visible with the usual illumination, but, in all the samples examined, no organisms could be found that could not be seen by the ordinary microscope. One of the interesting observations, however, was that some Azotobacter (one of the soil bacteria) appeared in the filtrate, although this organism is at least 5 microns in length in a developed condition. This fact strongly suggests that this organism must have some stage when it is in a gonidial condition, as suggested by Löhnis and Smith,[10] and, consequently, is so much smaller in size that it could pass through the 1 micron pores of the filter. This work of Miehe's led him to suggest that the ultramicroscopic organisms must be very scarce in nature.

A similar lack of chemical change and of organisms in soil water passed through a filter had been recorded by Rossi,[20] although Melin[12] says that ultramicroscopic forms exist in forest soil.

The evidence seems to be entirely negative; an important and independent living world does not lie beyond the range of the microscope, or, if such organisms exist, they would seem to be rare.

Errera[5] arrived at the same conclusions from the theoretical standpoint. A hypothetical micrococcus (one of the spherical organisms) with a diameter of only 0.1μ would contain, at the most, 10,000 protein molecules, while one with a diameter of 0.05μ would contain only 1,000 molecules, and it is doubtful if a living organism can be maintained with this number. Miehe's estimate of the smallest organism visible with the best lenses, is a spherical coccus, about 0.2 micron in diameter with 15,000 to 50,000 protein molecules.

Errera believes that there may be organisms one hundred times smaller than the usual bacteria but that these invisible forms can be seen or almost seen, since they are not so very much more minute than the smallest known organisms. The ultramicroscopic forms may be able to exist independently for a time but cannot grow nor reproduce; they must be in union with some other organism.

Ultramicroscopic Viruses.—Beyond the bacteria that can be seen with these powerful lenses, there are other organisms that are so small they escape the keenest eyes. They are known only by their activity; for instance, they, in many instances, can pass through the finest filters, filters made, with pores so small that organisms visible under the microscope are prevented from passing through them. It is known that these *ultramicroscopic organisms* pass through filters because from the filtrate can be obtained the same results as were produced by the material before it passed through the filters, *i.e.* the production of the disease. Many diseases are produced by organisms, such as typhoid fever, cholera, and anthrax bacteria, which are large enough to see and will not pass through a Pasteur filter (very fine-pored, unglazed clay) into the filtrate. On the other hand, some of the commonest diseases, such as smallpox, foot-and-mouth disease, hog cholera and tobacco mosaic, are caused by ultramicroscopic organisms, which will pass through such a filter. These are known as *ultramicroscopic viruses.*[9]

Filterable Viruses.—If some of the aqueous material from the diseased portions of a cow suffering from foot-and-mouth disease or of a tobacco plant affected by mosaic is passed through a Pasteur filter, no organisms will be visible in the filtrate and none could have been seen in the tissues of the animal or plant, even with the very best lens. The filtrate from the cow will, however, produce the same disease in another animal and the filtrate from the tobacco plant will induce mosaic. Among the diseases believed to be due to filterable viruses are hog cholera, cattle plague (Rinderpest), foot-and-mouth disease, smallpox, cowpox, swinepox, distemper of dogs, warts, mumps, measles, mosaic disease of various plants, infectious chlorosis, leaf roll of the potato, and, possibly, grippe, colds, and scarlet fever. The latter group is doubtful and many investigators now believe that these diseases may be due to small bacteria.

The causative agent slips through the pores of the filter and is still alive and capable of producing its particular disease. These organisms which pass the filter have been found to be uniformly impossible of further cultivation (if we except the work of Flexner and Noguchi[6]), possibly due to their extreme parasitism. They seem to lack some of the physiological properties of larger visible types so that the computations made by Errera may be correct since these types are, in themselves, not self-contained and perpetuating.

Duggar and Karrer[3] estimated the size of the infective bodies in tobacco mosaic to be 30$\mu\mu$ in diameter; made up of colloidal particles, probably with no real bordering membrane and no internal metabolism.[4] They must live in union with the cells of some other more complex type in order to continue to exist and for this purpose it may be conceived that each of them has some form of chemical attachment. Such bodies could not live free and independent, so they are never found in nature as saprophytes, as Molisch has pointed out. Work on scarlet fever would further indicate that these bodies, in some way, activate certain organisms such as *Streptococcus pyogenes*, which are found associated with the disease but alone will not produce it.

Other estimates of virus size vary between 20 and 200$\mu\mu$ but any figures are pure conjectures, as are those of protein molecules, and have a wide range.

The difficulty of cultivating viruses is generally admitted and their growth in the absence of living cells is very doubtful. They must be studied in the presence of living body cells and, in view of their ultramicroscopic size, the task is an almost impossible one. The only facts which can be determined are their effects on cells which include them.[18, 19]

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CHAPTER III

THE GENERAL METHODS USED IN MICROBIOLOGY

Microbiology is largely founded on the use of *pure cultures*, growths of the organisms on a nutrient substance in which only one variety is present. The earlier investigations were usually conducted on *mixed cultures*, so that the results which were obtained before the organisms were isolated into a growth of one variety have had to be largely discarded.

The Principles of the Pure Culture Method.—Pure cultures are obtained by *dilution* and *plating out*. The credit for devising this combination is usually given to Robert Koch, but the dilution method seems to have been first suggested by Klebs,[4] and solid media by Schroeter,[9] although Koch was so successful in obtaining pure cultures, especially of the disease germs, that his fame has overshadowed the originators.

Cohn and other investigators had used slices of potato and other vegetables for the growth of microorganisms, but Schroeter grew the color-producing bacteria on coagulated milk, starch paste, flour paste, and the white of egg, cooked and uncooked; this last substance is especially favorable for their growth. None of these substances had the peculiar advantage, however, of the substratum suggested by Koch, *gelatin*, which was liquid at very low temperatures, solid when cold, and very transparent, so that Koch's contribution was a real advance.

Ten per cent of gelatin added to beef broth will liquefy at a temperature of about 30°C. so that it cannot be used in incubators kept at 37°C., the blood or body temperature at which the pathogenic organisms of human beings and warm-blood animals usually grow best.

Agar.—The use of agar for thickening culture media is credited to Frau Hesse, apparently a laboratory assistant with Koch, who found that the red algæ would yield a substance which could be used for hardening broth containing meat extract and peptonized meat. It is so superior to gelatin that, since its introduction in 1881, it has almost entirely displaced gelatin for routine work. This product of the red seaweeds is imported from Japan and the eastern shores of the Pacific ocean in the form of shreds (shredded agar) known also as *agar-agar*. It serves simply as a carrier of food substances since very few of the bacteria and molds which grow on agar culture media are able to render it soluble. A solution of about 5 grams of peptonized meat (peptone) and 3 grams of meat extract in 1,000 cubic centimeters of water is thickened by the addition of about 1.5 per cent of agar shreds. This colloidal jelly hardens at about 37°C. and melts at about 93°C., so that while necessary to boil it to get it quickly into a liquid state, it will not harden until its temperature is about 37°C., a very valuable property as the majority of the microorganisms are killed, when not in a spore stage, at about 50 to 60°C.

The use of dilution methods for separating the bacteria so that a small number could be obtained in a drop of a substance had already

been devised by Klebs,[4] and the first pure culture known was obtained by the use of this method by Lister[6], who isolated the lactic-acid organism by repeated dilutions in sterilized milk until he was able to say that a single organism was in a tube of the dilutions and that such a tube was a pure culture.

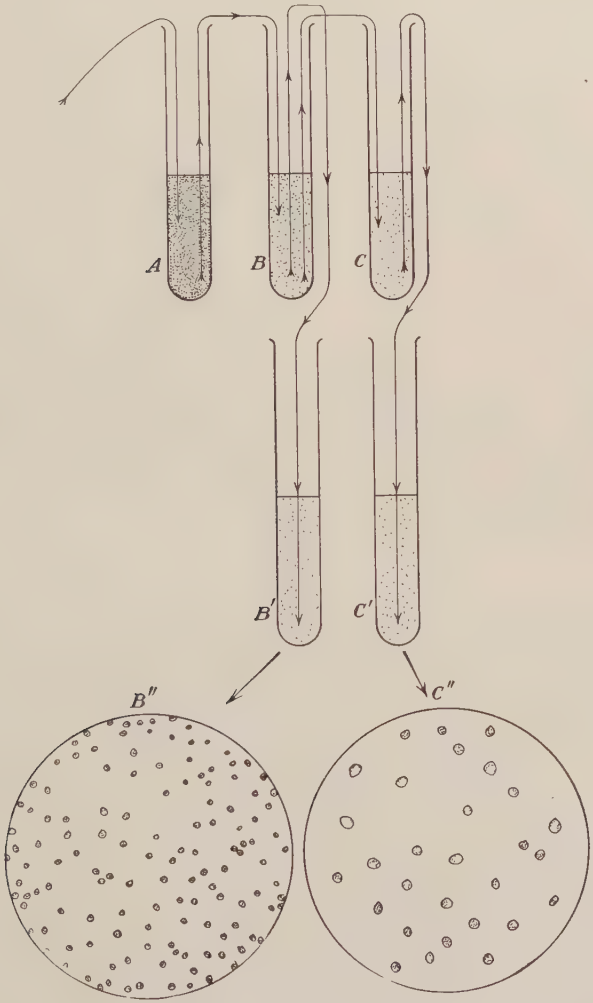

FIG. 19.—Diagram to show the dilution and plating method used in microbiology.

The combination of gelatin plates by Koch[5] together with the Kleb's dilution preliminaries was the greatest single advance in the technic of microbiology.

Petri suggested the use of the little individual dishes for the growth of cultures in 1887.

The modern methods for the making and sterilization of agar media are the results of the suggestions of a number of investigators, each of whom offered some

small improvement in order to render the agar constant in composition, clear, and without a precipitate.

The Plating-Out Method Outlined.—The necessary dilutions are obtained by transferring small portions of the material to be plated out to tubes of sterile water or agar, shown diagrammatically in Fig. 19. Two or three transfers are made into sterile water and then into melted agar at about 45°C.

The number of organisms in a drop of the liquid in this last tube is very small, so that, if the liquid is poured into a sterile Petri dish or plate (Fig. 20), they are scattered over quite a large surface.

If each of these organisms gives rise to a colony, the number of colonies will be small, of course, and one of them can be picked up with a sterile platinum needle and transferred to a sterile medium to grow into a *pure culture*. This is known as "plating out," since Koch used pieces of glass, about 12 to 15 centimeters in length and 8 to 10 centimeters in width, on which to pour the liquefied gelatin, carefully spreading it with the lip of the test tube in which it was contained, so that it would not run over the edges of the glass plate. These glass plates with the media on them were then piled up under a bell jar and separated from each other by strips of glass under the ends of each plate. The introduction of the small individual glass dishes for each culture was an improvement, since the operation of plating out, as originally followed, had to be done in a room free from dust in the air, and the most elaborate precautions had to be taken to prevent the entrance of extraneous organisms into the gelatin.

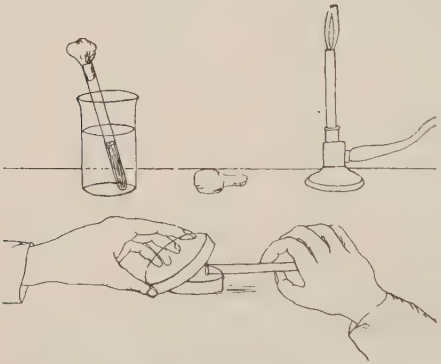

FIG. 20.—Pouring a plate of gelatin or agar into a Petri dish. (*From Löhnis, Laboratory methods in agricultural bacteriology.*)

These plating-out methods are very simple, but on them the initial success of modern bacteriology has depended, for, by the use of this technic, Koch and later investigators were able to isolate and study pure cultures of bacteria, something which was practically unknown before 1880.

Enriching the Culture before Plating.—Other devices have been introduced to obtain pure cultures easily. For almost every organism some special treatment is advisable in order to obtain, on the isolation plates, a large percentage of the desired species. Sometimes, boiling will destroy all except the one desired, if the latter happens to be a spore-forming organism; with another, a particular type of medium will give it an advantage over the organisms by which it is surrounded and with which

it must compete; with another, high temperature for incubation may favor its growth at the expense of other forms.

Defects in the Method.—It is necessary to caution overenthusiastic users of the plating-out method, however, that it will not necessarily always yield pure cultures. Two organisms may fall so close to each other that the colony which develops is a mixed one and the mixture cannot be detected by examining the plate. Miquel,[7] in examining the air in and around Paris for the number of organisms which could be found on gelatin plates exposed to contamination, found that out of 442 colonies which appeared, 385 were bacteria; but only 136 contained one variety while 87 contained two, 75 contained three, and 87 contained four or more species. This count is probably hardly comparable to that obtained from the ordinary plating-out method, however, since these colonies came from organisms which were borne on bits of dirt and two or more might readily find lodgment on a single piece. On the other hand, it is questionable whether any shaking will completely separate clumps of bacteria into individual bacteria so that it can be said definitely that one colony is composed of the descendants of one organism. Many of the colonies come from clumps of the same organism.

OTHER METHODS FOR OBTAINING PURE CULTURES

Since the plating-out method gives no opportunity for again locating on a culture plate any particular organism seen under the microscope,

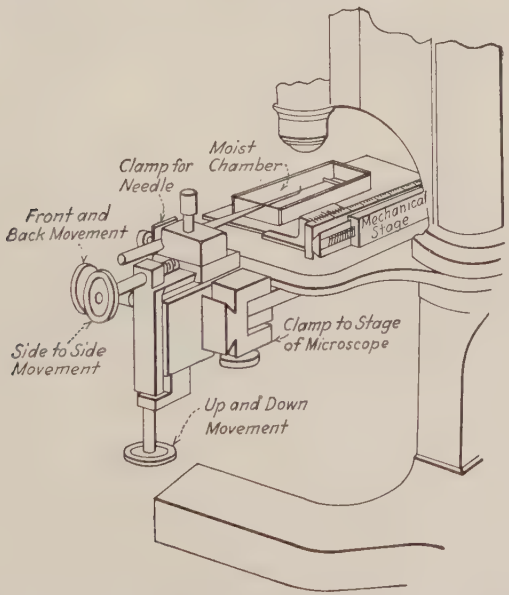

FIG. 21.—The Barber apparatus for picking up individual bacteria.

various attempts have been made to devise an apparatus to pick up any desired specimen as seen under the high power lens of the microscope and from it start a pure culture. In view of the extremely small size of the bacteria such an apparatus must be very accurate.

Hansen,[3] as described later, succeeded in obtaining single-cell cultures of yeasts, but the method does not seem to be easily applicable to the bacteria.

The isolation of objects as minute as bacteria was accomplished successfully by Barber[1] by the use of a capillary pipette moved mechanically by means of screws, operating somewhat in the manner of a mechanical stage (Fig. 21).

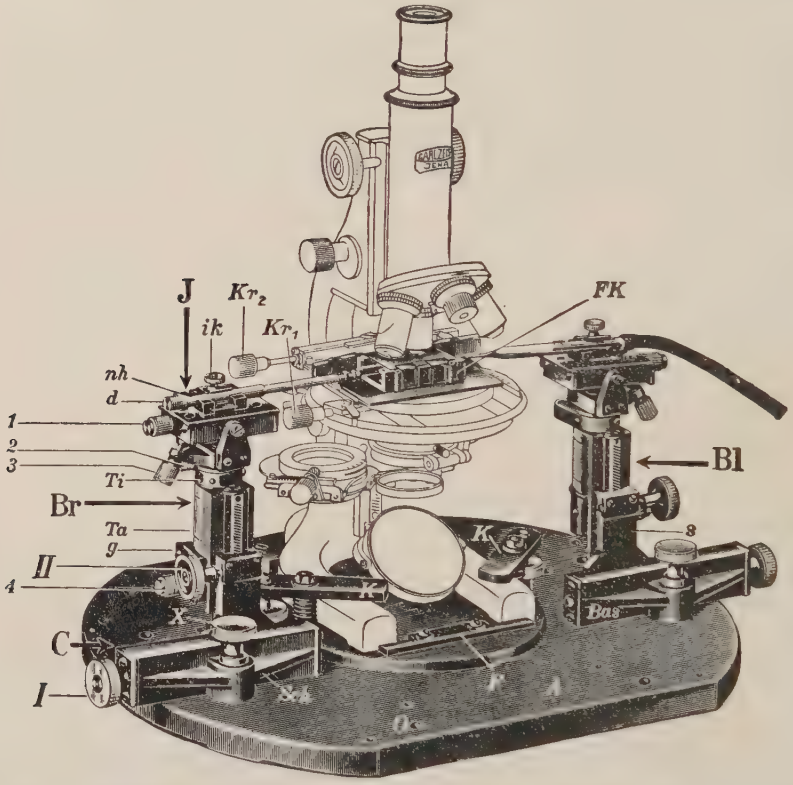

FIG. 22.—A manipulator for picking up individual bacteria; for this work only one of the needles is necessary. (*From Carl Zeiss Company.*)

The organisms suspended in a hanging drop are examined under the high-power lens of the compound microscope. A very fine capillary pipette, manipulated from the side by means of thumb screws, is brought *under*, but not *into*, any part of the hanging drop. A particular type of microorganisms can be selected from the drop, the pipette brought up into the drop, and the particular organisms sucked up into the fine tube of the pipette. The small drop in the pipette may then be moved to another part of the cover glass, emptied, and a reselection made. The contents of the pipette containing the single cell may then be transferred to an agar tube or plate and grown in a pure strain.

The Barber apparatus has been further improved by Chambers,[2] the movement of the pipette being in the form of arcs through the field instead of the straight right-and-left, back-and-front movements of the original machine. Two needles have been provided, also, so that cells can be dissected under the microscope. Several scientific apparatus makers have put various types on the market, one of which is illustrated in Fig. 22.

The advantage of such isolations made by the pipette method over those obtained by plating out is that the individual can be observed under the microscope before it is transferred to an agar medium. Barber, as will be noted later, obtained, by the use of his apparatus, results which would have been practically impossible by the ordinary technic. Other methods have been suggested and used by individual investigators.[8]

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CHAPTER IV

MICROORGANISMS AND THE ORIGIN OF LIFE

The small size and simplicity of structure of the microscopically visible microorganisms, particularly the very small spherical forms, led observers, when they first became familiar with them, to surmise that science had found the connecting link between the organic, or living world, and the dead, or inorganic world of rocks, soil, and crystals. The sudden appearance of this form of life in organic solutions such as beef broth, on cooked vegetables, and foods seemed to indicate that all that was necessary for their appearance was the proper nutrient substance; Was Harvey's *Omnes vivum ex vivo* incorrect? It must be recalled that this theory that living beings could originate from lifeless chemicals was not a new one but was a survival of the older belief in the spontaneous origin of life. The two schools of thought were (1) the believers in *biogenesis*, life only comes from preexisting life; and (2) the believers in *abiogenesis*, life can originate without preexisting life from chemicals or the dead bodies of plants or animals.

The Spontaneous Origin of Animals.—The sun's rays were supposed to have a very active effect in starting life from rich deposits of mud or slime, especially under damp conditions. The mice which swarmed in the Nile valley, after the spring floods had left a deposit of rich humus over the fields, were cited as an uncontroverted example of such spontaneous origin for one of the higher animals.[2]

The appearance of maggots in meat, derived from the deposition of eggs of the flies, as we now know, was held to be spontaneous until an Italian physician, Redi, (1626 to 1697) showed that he could prevent their development by simply covering the meat in such a manner that the flies had no access to it. Meat was placed in jars, some of which were left uncovered, some covered with a fine wire screen, and others covered securely with parchment. The meat decayed in all the jars, but only in open jars where the flies could lay their eggs in the meat or in those where they could sit on the screened top and deposit eggs through its meshes to the meat below did any maggots develop. The maggot was simply another stage in the life of the fly and if the fly was not present, the larvæ (maggots) would never appear.

Needham's Experiments.—The discovery of bacteria and their wide spread in nature introduced an entirely different phase. The appearance of these organisms in cooked food was very suggestive that

they arose from the food itself. Experiments made by an English priest, Needham (1713 to 1784), who boiled meat juice in vials which he afterwards corked carefully and sealed, and boiled a second time, showed the vials, in the course of time, becoming cloudy from the growth of bacteria which had apparently originated from the meat juice itself.

Spores in the Bacteria.—One fact with regard to the bacteria must be remembered at this point, since all this experimentation hinged upon it, although, at that time, it was entirely unknown. The bacteria, as well as the other microorganisms, are killed by a single boiling as these experiments had proved, in a way, but this temperature was not sufficient to destroy the small but very thick-walled and resistant *spores* which were enclosed inside many of the cells. These spore-bearing bacteria are very common in soil and air and might readily find their way into any meat juice, placed in a flask, unless the most elaborate precautions were taken. *A single boiling will not kill these spores but intermittent boilings will.* After they emerge from this resistant spore stage into the ordinary or *vegetative* phase, they may be readily killed. The importance of the spores in the bacteria was not discovered until about 1871, by Cohn, so that this early experimental work was done without that knowledge. With this fact in mind, it will be interesting to follow the further development of the experiments to prove or disprove the spontaneous origin of life.

Spallanzani's Experiments.—Needham's experiments were repeated by other experimenters with similar results, so that his theory was generally accepted. Abbe Spallanzani, an eminent Italian natural scientist, professor at Bologna and afterwards at Pavia, called them into question, however; the basis of his objections being that the flasks were not clean before the meat juice was introduced into them. He confirmed Needham's experiments, showing that even boiled liquids become cloudy from the growth of microorganisms, and then set to work to explain the reason for their appearance. He heated the flasks into which the meat juice was to be introduced to such a temperature in the fire that he was certain that he had destroyed all living things before he filled and sealed them. These hermetically sealed flasks were boiled for an hour and then kept indefinitely without any cloudiness or bacteria appearing. If, however, the flasks were not sealed, the bacteria would make their appearance in a few days.

This method of preserving foods was soon put to a practical use by Appert, in Paris in 1806, and is the basis of all the modern canning of foods.

Other Experiments Using Different Methods.—This experiment of Spallanzani's, however, did not stop discussion of the origin of these lower forms from lifeless material. The objection was soon raised that he had altered the constitution of the air in the flasks by long boiling

and that, as no new air could enter the flasks, the organisms could not start; fresh air must be present since it contained the real life-giving principle. The solution of the problem now turned to attempts to admit air and still preserve the fluid sterile inside the flasks. Some ingenious devices were proposed by various experimenters to avoid this very real difficulty and objection. It will be noted that the use of cotton or wool to stop test tubes or flasks was not generally understood, although Schroeder and Von Dutch had shown, in 1854, that filteration of air through a layer of either of these substances would free it from all living organisms.

Schulze boiled his meat juice as had Spallanzani, but, instead of sealing the flask, connected it with the outside air by means of a glass tube in which were two coils, one containing concentrated sulphuric acid and the other a potassium-hydroxide solution. The other side of the flask was attached to an aspirator so that new air could be introduced into the flask after it had passed through the sulphuric acid and potassium hydroxide solutions. The air came through in bubbles so that he was very fortunate in not introducing some organisms into the meat juice, and no growth appeared.

Schwann tried a similar experiment but passed the air not through strong acids and alkalies but through a heated tube, depriving it of this "vivifying principle" which made possible the development of the living organisms.

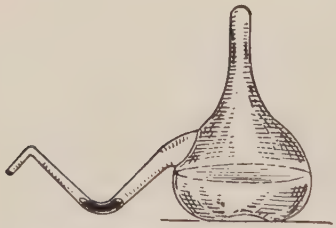

FIG. 23.—Flask used by Pasteur in experiments on spontaneous generation. (*From Shull's "Principles of Animal Biology."*)

Pasteur Uses a Special Flask.—The question was finally settled, so far as the spontaneous origin of life was concerned, by the use of meat extract in a sterile flask, boiled and allowed to remain; the new feature being that the flask used was drawn out into a long curved tube known as a "goose neck." The steam could pass out of this goose neck and the air could enter but the bacteria could not. They would be caught in the long curved tube (Fig. 23).

The invention of this flask is usually credited to Pasteur (although he seems to have been superseded by Hoffman and Chevreul) who used it effectively in his demonstration and famous lecture before the Paris Academy of Science[3] delivered Apr. 7, 1864:

Here is an infusion of organic matter as limpid as distilled water, and extremely alterable. It has been prepared today. Tomorrow it will contain animalculæ, little infusories, or flakes of moldiness.

I place a portion of that infusion into a flask with a long neck, like this one. Suppose I boil the liquid and leave it to cool. After a few days, moldiness or animalculæ will develop in the liquid or against the glass; but the infusion being

again in contact with air, it becomes altered, as all infusions do. Now suppose I repeat this experiment, but that before boiling the liquid, I draw (by means of an enameller's lamp) the neck of the flask into a point, leaving, however, its extremity open.

This being done, I boil the liquid in the flask, and leave it to cool. Now the liquid of this second flask will remain pure not only two days, a month, a year, but two or three years. The experiment I am telling you about is already four years old, and the liquid remains as limpid as distilled water. What difference is there, then, between those two vases? They contain the same liquid, they both contain air, both are open. Why does one decay and the other remain pure? The only difference between them is this: in the first case, the dusts suspended in the air and their germs can fall into the neck of the flask and arrive into contact with the liquid, where they find appropriate food and develop; thence microscopic beings. In the second flask, on the contrary, it is impossible, or at least extremely difficult, unless air is violently shaken, that dusts suspended in the air should enter the vase; they fall on its curved neck. When air goes in and out of the vase through diffusions or variations of temperature, the latter never being sudden, the air comes in slowly enough to drop the dusts and germs that it carries at the opening of the neck or in its first curves.

This experiment is full of instruction; for this must be noted, that everything in air save its dusts can easily enter the vase and come into contact with the liquid. Imagine what you choose in the air—electricity, magnetism, ozone, unknown forces even, all can reach the infusion. Only one thing cannot enter it easily, and that is dust, suspended in air. And the proof of this is that if I shake the vase violently two or three times, in a few days it contains animalculæ or moldiness. Why? Because the air has come in violently enough to carry dust with it.

I wait, begging it recommence for me the beautiful spectacle of the first creation but it is dumb because I have kept from it the only thing man cannot produce, the germs which float in the air, for life is a germ and a germ is life. Never will the doctrine of spontaneous generation recover from the mortal blow of this simple experiment.

The theory of spontaneous generation did not recover although Dr. Bastain[1] long continued the fight for the origin of living beings from simple organic or inorganic matter. The scientific world decided, however, that the question was settled and all interest in it has been lost. "All life comes from life."

The Application of Pasteur's Discovery by Lister.—These experiments of Pasteur, in 1864, in which a purely theoretical question was discussed and decided, gave rise to some of the most important practical applications that can be conceived in their complete revolution of the practice of surgery, showing that an experimenter can never know the application to which his apparently useless results may be put.

Dr. Joseph Lister (Fig. 24) was professor of surgery in charge of one of the wards of the Glasgow Infirmary. He discovered, from Pasteur's work, the importance of the germs borne on the dust particles in the air, and decided that, for surgery to be successful, it must either exclude

these germs or put something into the dressing of the wound which would kill them or prevent their growth. The disease known as "gangrene" was rampant in every hospital in those days, and more patients died from it than from the shock of operations. Lister suggested soaking the bandages used in the dressings in a 5 per cent solution of carbolic acid, and in a few months his wards showed the lowest death rate of any hospital in the world. The use of antiseptics and the care now practiced in preventing infection have made possible complicated and delicate

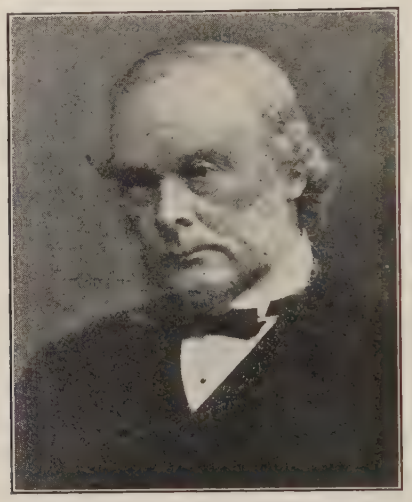

FIG. 24.—Sir Joseph Lister (1827–1912). Originator of antiseptic surgery.

operations upon almost every organ in the body; before we knew the danger of dust and germs they would have been absolutely impossible, for not a single patient would have survived the subsequent infections. The relation of bacteria to disease is discussed in a later chapter.

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CHAPTER V

INORGANIC CHEMICAL ELEMENTS ESSENTIAL FOR THE GROWTH OF MICROORGANISMS

The general requirements of the colorless plants, in the form of water and nutrient salts, are not materially different from those of the higher green plants or the algæ. The analysis of the ash of higher plants gave the clue as to the mineral constituents which the plant absorbed, and various combinations have been suggested by plant physiologists to meet those requirements. The labors of Liebig and his students during the years 1830 to 1870 furnished a mass of data on a wide range of plants which was summed up by Wolfe.[36] The plant chemist, using distilled water and pure chemicals, made up "water cultures" which simulated soil water, and in which he was able to grow flowering plants, such as the sunflower, corn, oats, or buckwheat, to maturity.

Chemical Combinations for Higher Plants.—Altogether, over 30 of the elements have been found in the ash of higher plants,[19] but the majority of the suggested media contain only 7 of them: *nitrogen*, *phosphorus*, *magnesium*, *potassium*, *calcium*, *sulphur*, and *iron*. The following solutions may be taken as representative:

	Knopf[12]	Pfeffer[21]	Tottingham[29]	Shive[25]
$Ca(NO_3)_2$	1.0 grams	0.57 grams	2.36 grams	0.85 grams
KNO_3	.....	0.14 grams	0.5 grams	
$MgSO_4$	0.25 grams	0.14 grams	1.74 grams	2.41 grams
KH_2PO_4	0.25 grams	0.14 grams	1.76 grams	2.45 grams
KCl	0.12 grams	0.07 grams		
$FeCl_3$	Trace	Trace	$Fe_2(PO_4)_2$ Trace	$Fe_2(PO_4)_2$ Trace
Water	1,000 cubic centimeters	1,000 cubic centimeters	1,000 cubic centimeters	1,000 cubic centimeters

It will be noted that Tottingham reduced the number of chemicals to four, and that Shive brought the number down to three, claiming that wheat and buckwheat thrive in this limited number. These solutions are very dilute, as are the soil solutions, having an osmotic pressure in the two latter solutions of about 2.50 atmospheres.[25] The cell sap of higher plants does not have a high osmotic pressure and the solutions used in water cultures must have much less. In such solutions,

wheat, corn, and buckwheat can be grown and seed harvested; no other constituents seem to be necessary.

Inorganic Chemical Combinations for Microorganisms.—With microorganisms, the same, or a similar series of chemical compounds are required, and numerous series of such combinations have been tested. The first solution compounded, however, used simply the ash of one of these lower forms without endeavoring to analyze it or to substitute inorganic chemicals for those which had been built into the plants. The use of this original solution of microorganisms grew out of the controversy between Liebig and Pasteur over the fundamental nature of a fermentation, such as occurs when yeast is added to a sugar solution.

Pasteur's Ashes-of-yeast Solution.—Some authors had claimed that the lower forms, such as the yeasts, were composed of pure carbon compounds in the form of carbohydrates and proteins and that, if such a body were ashed, every trace of it would pass off into the air as carbon dioxide. According to Liebig's theory, too, the albuminoids were necessary for fermentation, since it was by the destruction of these compounds that the yeast or fungus was able to build its protoplasm. Pasteur[20] prepared a solution in which yeasts would grow without any proteins, the necessary salts being derived from the ashes of yeast:

Water	100 cubic centimeters
Ammonium tartrate	1 gram
Cane sugar	10 grams
Yeast-ash	0.075 gram representing the ash of 1 gram of pure yeast

Pasteur grew yeasts and fungi on this solution as well as though proteins were present. He later prepared another solution, "yeast-water," which he used for the same purpose and also for the growth of bacteria. This solution was made as follows: 100 grams of brewer's yeast, or 75 grams of compressed yeast, were boiled for about 15 minutes and then filtered through paper. The pale-yellow, clear filtrate obtained was made up to 1 liter and sterilized. Many of the proteins of the yeast must appear in this solution so that it cannot be regarded as a synthetic medium. With the addition of acetic acid to the yeast water and a small per cent of alcohol, Pasteur adapted it to the culture of the acetic-acid bacteria.

Synthetic Inorganic Combinations.—The ashes of yeast are not readily soluble, and, in order to remedy this defect, Adolph Mayer[16] compounded a solution of salts which contained, as he believed, all that was necessary for the growth of colorless organisms. His solution is especially interesting since it seems to have been the first real attempt to combine salts necessary for growth of colorless plants, according to the analysis of their ash. It was made as follows:

Water	1,000 cubic centimeters
Cane sugar	150 grams
Ammonium nitrate	10 grams
Monopotassium phosphate	5 grams
Magnesium sulphate	2.5 grams
Calcium phosphate ($CaHPO_4$)	0.5 gram

Another of these older solutions which has been used extensively is that of Raulin[22] made as follows:

Water	1,000 cubic centimeters
Cane sugar	47 grams
Tartaric acid	2.7 grams
Ammonium nitrate	2.7 grams
Ammonium phosphate	0.4 gram
Potassium carbonate	0.4 gram
Magnesium carbonate	0.27 gram
Ammonium sulphate	0.16 gram
Zinc sulphate	0.05 gram
Ferrous sulphate	0.05 gram
Potassium silicate	0.05 gram

It would seem from the above recipe that Raulin tried to put into his solution everything that he thought might possibly be needed by the fungus. His formula, although very complex, was based on a series of trials of various salts to determine whether or not they were indispensable.

Cohn[5] used as a liquid in which to grow bacteria a solution composed of:

Water	100 cubic centimeters
Acid potassium phosphate (KH_2PO_4)	0.5 gram
Calcium phosphate $Ca_3(PO_4)_2$	0.05 gram
Magnesium sulphate crystals	0.5 gram
Ammonium tartrate	1.0 gram

Uschinsky's solution[30] was made to grow some of the disease-producing bacteria to determine whether they would produce their toxins in a purely synthetic medium. He claimed to be successful in developing toxins from the diptheria and tetanus organisms with the following combinations:

Distilled water	1,000 cubic centimeters
Glycerine	30 to 40 cubic centimeters
Sodium chloride	5 to 7 grams
Calcium chloride	0.1 gram
Magnesium sulphate	0.3 to 0.4 gram
Dipotassium phosphate	2 to 2.5 grams
Ammonium lactate	6 to 7 grams
Sodium asparaginate	3 to 4 grams

Richards' solution[23] for growing fungi has been widely copied and modified; his basic solution was as follows:

KH_2PO_4	0.50 gram
$MgSO_4$	0.25 gram
NH_4NO_3	1.00 gram
$FeSO_4$	trace
Cane sugar	5 grams
Water	100 cubic centimeters

This solution was modified in various ways by substituting other sources of nitrogen and carbon, such as 5 grams of asparagin for the ammonium nitrate; 1.0 gram of peptone instead of the ammonium nitrate; and, in one solution, 5.00 grams of glycerine and 1.00 gram of peptone as sources of carbon and nitrogen.

The Essential Difference between Solutions for Fungi and for Green Plants.—A careful examination of the culture solutions for green plants and those for fungi and bacteria reveals one essential difference. The latter types of plants require some form of carbohydrate while the former is well fed if given only inorganic salts. The carbon, later to be used as a source of building material or burned as fuel to supply heat and energy as well, will be explained more in detail. Over 2,500 different combinations of inorganic salts have been proposed for microorganisms and in one set of experiments, Krosnow, Rivkin, and Rosenberg[13] tried out 617 modifications. The inorganic elements are about the same in all combinations, however, and it may be well to look at them in detail.

The Use of the Various Elements.—Not much is known definitely of the use which the various elements added to the culture solutions have for the growth of the higher plants placed in them and the same obscurity veils the destination of the chemical elements absorbed by microorganisms. Falk[7] has brought together a list of the literature on the rôle of the various ions. At least 17 different elements have been suggested as essential and have been investigated, but only the more important of these elements will be considered, 6 of them being generally considered necessary for the growth of the microorganisms: *phosphorus*, *magnesium*, *sulphur*, *potassium*, *iron*, and *zinc*.

Phosphorus.—A chemical analysis of protoplasm shows that sulphur, phosphorus, and magnesium are always contained in some of the numerous forms of proteins which compose it. Phosphorus, especially, is a constituent of the nucleoproteins which make up an essential portion of the nuclear material; in order to build nuclei, therefore, phosphorus must be supplied.

The addition of the salts of phosphoric acid to a culture solution increases the amount of carbon dioxide produced, not only in the green plants,[15] such as wheat seedlings and Elodea, but also in fungi and yeasts. The phosphorus in these combinations seems to be associated with the *anaerobic* type of respiration (the respiration which occurs in the absence of air), and the best explanation of the fermentation which occurs in sugar solutions, where alcohol is produced, that it is an incomplete phase of respiration of the yeast plant. A recent theory explaining respiration assumes that the process takes place by the action of enzymes which are made active in the oxidation of the carbohydrates by a catalyzer consisting of a combination of iron with phosphates.[14]

Calcium.—Calcium does not seem to be necessary for the colorless plants and is omitted from many solutions, or added in very small quantities only, although some bacteria may require it.[9] Growth is larger and spore formation in some of the molds will occur only in solutions containing some calcium.[6]

Benecke[1] has shown that it is not essential to the growth of microorganisms, although in green plants it serves some purpose in the translocation of carbohydrates and unites with the soluble oxalic acid formed during growth to produce an insoluble

compound, calcium oxalate, which is deposited in special cells. With the lower microorganisms, any oxalates which are formed are secreted into the culture media, and, in some species, are there crystallized out.

Magnesium.—The spores of Penicillium will not germinate in culture solutions which are free from magnesium. Molisch[17] inoculated with Penicillium a culture solution (containing 500 cubic centimeters distilled water, 10 grams ammonium acetate, 0.2 gram calcium sulphate, 0.2 gram acid potassium phosphate (dihydrogen), and 0.005 gram ferric sulphate) which he had placed in ten flasks. After 10 days there was no trace of fungus development. But when to half of the flasks a little magnesium solution was added, there developed, at once, a thick covering of mycelium, while in the flasks with calcium but no magnesium, there was no growth, even after a month. The addition of 0.02 per cent of magnesium sulphate was sufficient to cause growth of mycelium and fructification in *Aspergillus niger.* Large quantities of magnesium salts in a culture solution are detrimental to growth but this toxic action can be neutralized by the addition of calcium. For many of the higher plants, the proportions of calcium and magnesium required in a culture solution in order to neutralize each other have been established as Loew's calcium-magnesium ratio.

Magnesium, in many cases, appears to be associated with the transport in plants of phosphate as a magnesium phosphate. Magnesium is always abundant in the young, rapidly growing parts of a plant both in a free form in solution and combined in the protoplasm. It seems to be necessary also for spore formation in the fungi and for color production in some of the yeasts.

Sulphur.—This element is essential to protein production.

Potassium.—Potassium seems to be essential in the green plants for carbon-dioxide assimilation and possibly the transformation of the carbohydrates. The latter function may be necessary at times in the colorless plants.[4]

Iron.—The older theory that green plants needed this element for the construction of chlorophyl and that the fungi, not having chlorophyl, did not, was disproved by Molisch's very carefully conducted experiments with the fungi in which he redistilled the water and recrystallized the sugar used in the media.

When one of the molds, *Aspergillus niger,* was grown in a culture solution freed from iron, the addition of iron sulphate in very small percentages increased the yield of fungus mycelium three to fivefold.

Weight of fungus in iron-free culture medium, milligrams	Weight of fungus in iron-containing culture medium, milligrams	Per cent of iron sulphate added
105	280	0.002
99	274	0.002
85	270	0.002
88	310	0.003
73	346	0.004

In addition to the above facts, which give only an idea of the amount of growth, it was noted further that in no instance did the fungus produce spores in solutions in which iron was absent, while the surface was black with them in all the cultures to which it had been added. The iron is evidently essential in fungus cells for growth and reproduction.

Wehmer,[35] on the other hand, considers the small amount of iron which is absorbed by the fungus and the small amount found in the analyses of the mycelium as an

indication that this element is not a foodstuff but a *stimulant* which serves to increase growth. It is difficult to draw the line between a *stimulant* and a *food;* it is possible that many of the inorganic elements, such as copper, which are considered to be stimulants are really necessary in small doses for the proper development of plants. Miss Brenchley[2] suggests that two hypotheses might be offered to explain stimulation: (1) that the stimulants are valueless as foods but act as catalytic agents by means of which food may be assimilated; (2) that the stimulants are themselves essential for complete nutrition and are themselves of food value. If either hypothesis is correct, these stimulating substances are essential for the best growth and development.

Warburg[32–34] has revived an older theory that iron acts as a catalyst in oxidation in the cells. The iron in some unknown combination with nitrogen is the catalyzer, absorbing and breaking up the bonds of the amino acids in the cell. In the oxidation of the carbohydrates such as starch and sugar, the iron combines with phosphates to serve the same purpose. In the oxidation of fats, the SH groups are substituted for the nitrogen. The iron is deoxidized and becomes one of the lower oxides. When the oxygen is returned, it can again become a catalyst; thus, a small quantity only is necessary, since the reaction may be repeated any number of times.

According to Warburg's theory, the use of anæsthetics decreases the rate of oxidation since the anæsthetics themselves will enter into the iron-nitrogen catalyst and destroy its action. But since very dilute solutions of anæsthetics accelerate oxidation in many cases, the theory has been criticized by Buchanan.[3] The plasma membrane may have much to do with oxidation activity in spite of the fact that an exciting chemical may never pass beyond it and enter into the cell itself to induce oxidation.

Zinc.—Zinc may not be more important than the 17 other elements which have been investigated, but it has been more carefully studied, perhaps, because it is necessary in such small but measureable amounts.

Raulin[22] added a zinc salt to his culture medium. Richards[23] claimed that the action of zinc would be similar to that of iron, lithium, cobalt, nickel, and the fluorides. These substances in quantity are poisonous but in minimal doses seem to exert a stimulus. Richter[24] found that, while it stimulated the absorption and accumulation of organic substances, it checked the formation of spores, in this respect being unlike magnesium which seems to be essential for their formation.

Javillier[10,11] found that this metal, although present in small quantities in most plants, as shown by their ash, seemed to have its greatest effect on the fungi. *A. niger* grew better in culture media to which it had been added, showing a marked increase in the dry weight; perhaps it served as a catalytic agent stimulating absorption.

The quantity of zinc necessary to produce this stimulatory effect has been found by different authors to be variable, as the following table from Steinberg[26] shows:

Milligrams of Zinc in 1,000 Cubic Centimeters

Raulin	10.61
Richards	9.10
Ono	8.41
Richter	0.09
Javillier	0.10

Causes of Varying Effect of Zinc in Cultures.—These results are so diverse that Steinberg[27,28] has reinvestigated the whole matter and arrived at the following conclusions:

Various strains of the *A. niger* vary in their optimum amount for stimulation. This investigator isolated two strains of this mold, one of which required much more

zinc to stimulate it than the other. The two strains, isolated from single spores, also showed some small cultural and morphological differences. With the sensitive strain, 0.05 milligram of zinc in the liter of culture solution would produce a maximum of stimulation in 1 week, while the other strain would require 0.1 milligram. In cultures kept for 20 weeks, 5.0 milligrams of zinc would give a maximum growth such as could be obtained with the sensitive strain with 1.0 milligram.

Acidity seems to have much to do with the sensitiveness to stimulation and an acid culture medium increases the sensitiveness of the organisms to the stimulative effect, as does the presence of traces of iron.

All investigators who have experimented with these elements, required in such small quantities, agree that it is almost impossible to get chemicals and glassware that are absolutely free from traces of the elements on which the experiments are being conducted and the requirements are so small that only a trace will suffice.

Steinberg[26] used flasks of various types of glass, some zinc free, such as Kavalier Bohemian and Pyrex, for comparison with others zinc containing, such as the ordinary Jena make. The fungus in its growth may dissolve sufficient zinc from the glass container to furnish the stimulus even without the addition of any zinc to the culture medium itself.

Traces of Many Ions May Be Necessary.—These investigations will suggest one conclusion that has been generally missed by students of plant nutrition. The ordinary formulæ for culture media of the synthetic type, where 7 to 9 elements are supplied, may be all at fault in the assumption that they supply the needs of the organisms. Such plants may require a long series of other ions for proper development but get the necessary traces of them from the medium or the vessel in which they are growing. The addition of small quantities of these substances to synthetic formulæ gives more nearly normal growth than is obtained in their absence and we consider the increased growth due to stimulation.

Growth in the Absence of All Inorganic Material Except One Element.—If the inorganic salts are entirely lacking and a mold is grown with only the carbon compounds, in vessels which contain one of the elements in the form of a metal, the question arises, can it grow and produce spores? Fermi[8] grew *Aspergillus niger* in pure distilled water, to which had been added 2 per cent of ammonium lactate (free from all mineral salts), in containers of iron, lead, copper, nickel, zinc, gold, silver, tin, and aluminum. Good development occurred in the iron vessels and the mycelium had a distinct red tint from the precipitate of iron salts in the cells. Strong growth and spore formation also occurred in vessels of gold, aluminum, tin, and porcelain, while no growth occurred in the zinc or silver vessels, and very little in the copper or nickel. If these experiments were accurately carried out (as they seem to have been) it would mean that these organisms can continue growth with only traces of the other inorganic salts in the substratum.

Change in Reaction of Used Culture Solutions.—The absorption of the salts from solutions often changes the reactions of the medium since the salt is not taken into the protoplasm as a molecule but in an ionized form. The plasma membrane exerts some choice in regard to ions

which are removed from the solutions and more of some are chosen than of others. If the ion, removed in excess, is the anion, the solution becomes alkaline in reaction; the nitrates, such as potassium or sodium nitrate in a solution in which these colorless plants are grown, lose the nitrate ion to the microorganisms and gradually become alkaline. On the other hand, in solutions where the kation contains the portion absorbed more rapidly, the solution becomes acid. If ammonium sulphate is used as the source of nitrogen in a culture solution, the plant uses the ammonia ions, rejects the sulphate ions, and the solution becomes acid by the addition of these ions to water to form sulphuric acid. These changes in reaction undoubtedly are the cause of a part of the effects which are obtained by using old culture media for the continued growth of molds or bacteria and will be discussed later. In addition, there are also various excretion products which are probably organic in nature and which also tend to make the old, used media unsuitable for a second crop of an organism.

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CHAPTER VI

THE CARBON CYCLE IN NATURE

Green plants are machines which catch and store a small part of the sun's energy which falls on them as sunlight. The green coloring matter (chlorophyl) which they hold in their cells in various shaped chloroplasts is a light sensitive chemical which has the ability to combine the carbon dioxide of the air with water from the soil to build up carbon compounds (Fig. 25). The first visible form of this stored food is starch, although plant physiologists have much evidence that it is preceded by formalde-

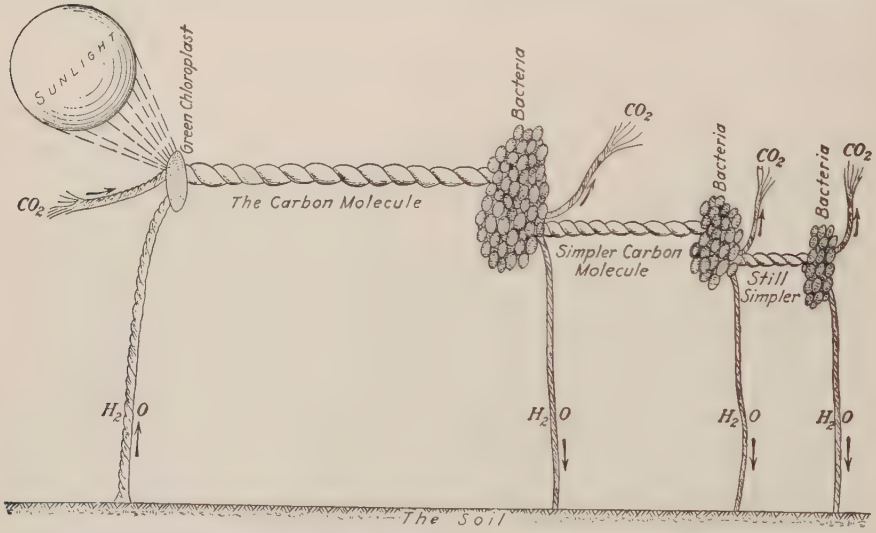

FIG. 25.—The building up of the carbon molecule from the CO_2 of the air and the H_2O of the soil. The molecule is represented as strand, twisted together under the action of sunlight and the green chloroplast; untwisted by microorganisms.

hyde and glucose. The starch may be later converted into soluble carbohydrates, such as sucrose, maltose, dextrose, or levulose, to be conveyed to various parts of the plant, or stored as starch, fat, or proteins. Whatever chemical form these carbon compounds may assume, however, they are to be regarded as containing stored energy, although the chemical formulæ by which they are known do not show it.

Man has devised sun engines to catch the radiant energy of the sun by mirrors, concentrating it on water in a closed vessel to make steam, but these clumsy and complicated machines are useless except in the

almost cloudless desert regions such as parts of California, Arizona, and the Sahara where the sunlight is constant and intense. The green plant is the only successful sun engine which will operate when the light is very weak and diffuse.

Even the green plants are not highly efficient as they utilize only about 1 per cent of the radiant energy which falls on mature, active leaves during the growing season.[10] Such plants are active during the summer months, but the sunlight which falls on the land in the winter time is not utilized. The very small proportion of the total solar energy which falls on an acre of land during a year which would be stored in a crop of 25 bushels of corn is shown diagrammatically (Fig. 26).

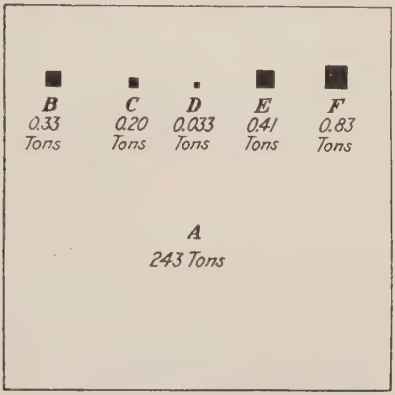

FIG. 26.—The amount of sunlight energy received by an acre of land is represented by the large square *A*. The energy absorbed is represented by a yield of 25 bushels of corn, square *B*; the energy of this corn converted into alcohol, square *C*; the energy obtained if the same corn is fed to animals (steers), square *D*; the energy stored by an acre of redwood in a growing season, square *E*; the energy obtained from an acre of Eucalyptus, square *F*. (*After Bruce.*)

The Stored Energy Is Released as Heat.—The trunk of a plant, such as a tree, may be utilized as fuel, releasing the stored-up sunlight of 50 years or more ago; or plants which have been converted into coal and buried under the earth for millions of years are brought to our fires to release for us the energy that fell on the earth when the world was younger. The point is well illustrated in the anecdote that is told of the great English engineer, Stephenson, who recognized this function of the green plants:

"One Sunday as people were returning from church, with Stephenson and Buckland among them, the whole company stopped upon the terrace beside Drayton Castle to watch a railroad train as it vanished rapidly in the distance, with a trail of white smoke behind it.

"'Well, Buckland,' said Stephenson as he turned to the famous geologist, 'answer a question, not a very easy one, perhaps. Can you tell me what sort of a force it is that drives yonder train along?'

"'Well,' answered the geologist, 'I should think that the force was one of your great engines.'

"'Yes, but what moves the engine?'

"'Why, one of your Newcastle engineers, of course.'

"'No, sunlight.'

"'How can that be?' asked the doctor.

"'I assure you it is nothing else,' replied the engineer. 'It is light that has lain stored in the earth for many thousands of years; the light absorbed by the plant during its growth is essential for the condensation of carbon, and this light,

which has been buried in the coal measures for so many years, is now unearthed and, being freed again as in this locomotive, serves great human needs.'"

Conversion of a Carbohydrate Means a Loss of Energy.—If the amount of energy which is accumulated by the green plants from the sun's rays is converted into alcohol or meat, there is an important loss in the conversion. In spite of these losses, however, mankind is very likely to demand much of its food in some other form than the carbohydrates directly produced.

Since *energy* is not the only character to be considered in food, however, a number of food plants are cultivated in which the energy storage is not so high but whose other properties are to be utilized, such as wheat for bread, or sweet potato for its flavor and sweetness.

THE COLORLESS PLANTS RELEASE ENERGY

While the green plants may be regarded as the world storers of the active energy of the sun, the colorless plants and the animals are the principal agents which release it.

The essential physiological functions of a green plant and a colorless one (without chlorophyl or chlorophyl in combination with other pigments), are, on the whole, very similar:

Green Plant	Colorless Plant (or Animal)
Intake of inorganic salts	Same
Photosynthesis with the production of starch and O_2	Absent
Assimilation to form other carbohydrates and proteins	Same
Respiration with the production of CO_2	Same
Transpiration and evaporation	Same

The essential difference, therefore, lies in the inability of the colorless plants to utilize the CO_2 of the air to form a stored carbohydrate with its latent, concealed energy. Nearly all of this type of plants, therefore, must be supplied with some form of energy-producing carbohydrate such as glucose, saccharose, ammonium lactate, ammonium tartrate, or glycerine, while the green plants can thrive on carbon derived from the air. An examination of the formulæ presented in the preceding chapter will show that carbohydrates or similar compounds are always present for the colorless plants to destroy and to release the energy. Exceptions must be made for the very primitive bacteria which are able to release sufficient energy for growth by the oxidation of ammonium salts, methane, sulphur, hydrogen sulphide, and possibly, iron.

Respiration in Colorless and in Green Plants.—Respiration means, chemically, nothing more than destruction, usually by oxidation, of some of the organic compounds. One of the simplest of these processes is in the destruction of glucose to form carbon dioxide and water:

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$$

Such an equation might represent the process of respiration inside one of the green plants, but would not be entirely accurate for the action of one of the colorless forms, since such complete oxidation rarely occurs from the action of one organism. One type of organism will carry the katabolic process to a definite stage, at which point it is poisoned by the action of the intermediate products which it has formed. Other and entirely different microorganisms may make use of the same slightly toxic substances for the further release of energy. The degradation of the carbohydrate takes place in a series of stages, in each of which a part of the contained energy is lost in the form of heat.

The Formation of Alcohol from Glucose by Yeast.—As an example of such a gradual destruction, the oxidation by series of organisms of a sugar (glucose) in cider or other fruit juices will serve. The organisms attacking glucose are numerous but the yeast plant is perhaps the most common. It breaks up the glucose into alcohol and carbon dioxide according to the following equation:

$$C_6H_{12}O_6 = 2C_2H_6O + 2CO_2$$

It is very probable that the equation is not as simple as this would indicate and that it takes place in a series of stages but, in any case, the important end products are alcohol and carbon dioxide. The ordinary yeast plants cannot tolerate over 10 per cent of alcohol; their growth is stopped by its toxic properties at this concentration and, since the alcohol content is dependent on yeast activity, the percentage remains constant. This stoppage of the growth of microorganisms from an excess of their by-products is a common occurrence.

The Formation of Acetic Acid from the Alcohol.—The further transformation of the alcohol is then taken up by some of the numerous acetic-acid bacteria. The equation in this transformation is as follows:

$$C_2H_6O + O_2 = C_2H_4O_2 + H_2O$$

This process is again one of oxidation to form a compound that has less energy value and is simpler in construction, the glucose containing 24 atoms in the molecule, the alcohol 9, and the acetic acid only 8.

The Destruction of the Acetic Acid by Mycoderma.—The destruction of the molecule is not yet complete, however, since acetic acid still contains some food value. The acetic-acid organisms cannot get this energy from the acetic acid but turn it over to a still different type, the so-called "Mycodermas." Mycodermas are forms intermediate between the yeasts and molds, budding like a yeast but developing a mycelium like a mold.[3,6] They are very abundant as a grayish-white scum on pickle vats where they gradually oxidize the acetic acid to carbon dioxide and water, the pickles meanwhile losing their sharp, acid taste and becoming soft. These organisms may build up other organic compounds and utilize some of the energy of the acetic acid in the formation of their

proteins, but the acetic acid, as such, is destroyed. Or, the vinegar eels (Nematode worms) may bring about the same change with a similar production of CO_2.

THE ENERGY LOSS IN THESE BIOLOGICAL REACTIONS

This description of the gradual oxidation and simplification of the complex organic molecule of sugar to water and a gas is, however, only one phase of the biological process. *Chemical equations* show only the kinds and numbers of atoms, together with the compounds in which they are assembled. No account is taken of the energy released in the various stages of simplification. *Energy equations* are as important in life functions as are those which only give the facts regarding the atoms and molecules, as they indicate the energy (heat) values of the compounds.

Every organic chemical contains within itself some energy of combustion which can be released by burning the substance and which can be very accurately determined by means of an instrument known as a "bomb-calorimeter." The essential feature of this instrument is that it holds the heat that is freed in combustion, and this heat is used to warm a definite measured quantity of water. One gram of the carbohydrate, or other organic substance, burned will warm 1 gram of water (1 cubic centimeter) a certain number of degrees; the number of degrees the water is raised in temperature giving the number of heat units contained and freed by burning. Thus, glucose, when burned, will give 3.76 of these heat units for every gram destroyed, ethyl alcohol, 7.08, and acetic acid, 3.50. It is customary, therefore, to say that glucose has a calory value of 3.76, (carbohydrates in general are assigned the value of 4), ethyl alcohol, 7.08, and acetic acid, 3.50. The word *calories*, as such heat units are termed, is not usually used in discussing stored heat but the large *Calory* (equal to 1,000 of the ordinary calories), the amount of heat necessary to raise 1 kilogram (1,000 cubic centimeters) of water 1°C.

It will be interesting at this point to trace the loss of heat in glucose through a series of reactions induced by various organisms, beginning with yeast:

$C_6H_{12}O_6$	$= 2C_2H_5OH$	$+ 2CO_2$
glucose	= alcohol	+ carbon dioxide
180 parts	= 92 parts	+ 88 parts
by weight	by weight	by weight
676.8 calories	= 651.36 calories	+ 0 calories

Putting the chemical equation in the form of an energy expression, the 180 parts of glucose multiplied by 3.76 have a calory value of 676.8. After the yeasts have acted on the sugar, the resultant alcohol, 92 parts with 7.08 calories, has a calory value of only 651.36 while the carbon dioxide has none. The loss in fermentation, therefore, is 25.44 calories or about 4 per cent of those contained in the glucose, representing the toll which the yeast has taken in its life processes. A part has been used

in the upbuilding of the proteins of the yeast; a part has been given off into the air as heat. A strongly fermenting vat of beer wort will show a rise in temperature of 10 to 15°F.[2]

The equation resulting from the activity induced by the acetic-acid organisms, when considered from the energy standpoint, would be as follows:

C_2H_6O	+	O_2	=	$C_2H_4O_2$	+	H_2O
Alcohol	+	oxygen	=	Acetic acid	+	water
46 parts by weight	+	32 parts by weight	=	60 parts by weight	+	18 parts by weight
325.68 calories	+	0 calories	=	210 calories	+	0 calories

It will be seen from this energy equation that of the 325.68 calories which entered into the equation, only 210 calories reappeared as acetic acid, representing a loss of 115.68 calories, or 35 per cent of the original value. The final destruction of the acetic acid by *Mycoderma*, or by vinegar eels, is less clear cut, but the final products have still less fuel value than even acetic acid, since the whole tendency of colorless plants or animals is to release the energy of organic compounds and convert it into heat and motion.

Colorless microorganisms such as the yeasts and molds, therefore, release some of the heat energy that has been stored by the green plants from the sunlight which fell on them. If the green plants were all destroyed, these colorless plants and the animals would die of starvation as soon as the store of food was exhausted. The only life that would survive would be the bacteria whose food and energy requirements are met by the oxidation of sulphur, iron, or simple chemicals not of organic origin.

The Relation of the Carbon Dioxide Produced to the Oxygen Consumed.—The volume of carbon dioxide produced should be the same as the volume of oxygen consumed for the oxidation of any of the carbohydrates, as may be seen from the following equation for glucose:

$$C_6H_{12}O_6 + 6\ O_2 = 6\ CO_2 + 6\ H_2O$$

The volume of the 6 parts of oxygen consumed would be the same as that of the 6 parts of carbon dioxide produced. The relation between the volumes of the two gases may be expressed as a *respiration quotient:*

$$\frac{CO_2}{O_2} = 1$$

Such a proportion is found not only in the algæ and flowering plants but also in many fungi and bacteria. The majority of plants vary in their respiration quotient, either above or below 1, depending on the conditions under which they were grown. The respiration quotient has been studied for a variety of plants but a change in the carbohydrate content of the substratum will alter it in a mold (*Aspergillus niger*):

TABLE 3.—RESPIRATION QUOTIENT $\frac{CO_2}{O_2}$ IN ASPERGILLUS,[9] WITH VARYING PERCENTAGES OF CARBOHYDRATES

Carbohydrate	1	1.5 to 2	3	5	10	15 to 17.5	20 to 25
Glucose	0.9	0.9	....	1.06	1.18	0.73	
Sucrose	0.87	....	....	0.96	1.02	.....	0.83
Raffinose	0.91	....	0.66				
Starch	0.68	0.55	....				
Glycerine	....	0.77	....	0.78	0.69		
Mannite	0.66	....	....	0.49	0.65		
Tannin	0.91	....	....	0.50	0.43		
Acetic acid	....	1.59	1.52	1.78	1.6		
Lactic acid	0.69	0.89	0.98				

The oxidation of foods, other than carbohydrates, causes the quotient to be less than one. The fats give a proportion of 0.7 and the proteins 0.81, but even such values are not usually obtained,[4] the respiration value[5] of peptone by *A. niger* being only 0.45.

Certain percentages in the solution of some of the carbohydrates give 1 and are apparently the optimum content for oxidation. If the fraction is very much smaller than 1, and carbohydrates are present in sufficient quantity, some of the carbon dioxide must be left in the cells to form organic acids. Two of the more common, oxalic and citric, are both formed in quantity by the mold, *A. niger*,[7,8] some strains producing as high as 30 per cent of oxalic acid, at times.[1] The formation of such acids is an indication that respiration is incomplete and that the carbon has been left as an intermediate product. Alcohol is probably such an intermediate product formed in quantity by the yeasts from an imperfect oxidation of sugar.

A fraction smaller than 1 may also mean that the organism is utilizing proteins or fats rather than carbohydrates or a combination of these substances with carbohydrates.

With the splitting up of nitrogen-containing foods, such as peptone, ammonia may be formed which may somewhat neutralize these acids. The salt formed may be nearly neutral and, as it still has much food value, be used in the building of proteins.

The formation of more carbon dioxide than can be balanced by the intake of oxygen indicates that some of the oxygen used in respiration must be derived from the rearrangement of the carbon compounds in the media, in such a manner that the new combinations contain *less* oxygen. Such conditions occur in a scarcity of oxygen, and other gases, such as hydrogen and methane, may be excreted along with the carbon dioxide.

The Oxygen—Carbon-dioxide Balance.—A certain amount of carbon dioxide seems to be of advantage to the cells of many of the bacteria, and

if they are freed from all traces of it, growth will cease. A certain amount of free oxygen, too, seems to be essential for the cell processes, particularly oxidation. The two gases for most organisms must be balanced, one against the other, and a certain optimum of each is best, both for growth and reproduction.[4] The carbon dioxide is probably not utilized as a source of carbon, however, but is of value in maintaining a chemical-physical equilibrium between the cells of the organisms and the medium surrounding them.

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CHAPTER VII

THE PRIMITIVE BACTERIA: THE SULPHUR, IRON, AND OTHER OXIDIZING SPECIES

When the heated earth had cooled from a temperature of hundreds of degrees centigrade to such a point that all the water was no longer held in the atmosphere as a vapor, but fell in torrential rains, great clouds of vapor enveloped the earth, shutting out the sunlight from the heated and drenched surface.

THE SULPHUR AND IRON BACTERIA

It was in this hot, humid, and almost sunless environment that life probably first appeared on the earth. Very few of the types of animals and plants found today could have existed at that time; only heat-enduring organisms could survive such conditions.[17] Sunlight was excluded, so the chlorophyl-bearing plants of all types would have found it impossible to survive and, since animals and the majority of the fungi and bacteria simply make use of materials stored in the bodies of the green plants, they could not have started off the long series of living organisms. Such a hypothetical ancestral organism must have used chemical means for acquiring the energy necessary for building up its simple body and for performing its life functions.[1]

Bacteria are still in existence today that can live, increase, and multiply on a purely chemical diet, the *sulphur* and, possibly, the *iron bacteria*, found in stagnant water, deriving their energy for life processes from oxidation. These organisms, in the oxidation of sulphur and iron, free energy even in complete darkness. Supplied only with inorganic salts and an abundance of this inorganic compound for energy, they are able to thrive as well as those requiring a more elaborate diet. On account of the simplicity and primitiveness of their food, Fischer[8] groups them together as the *prototrophic* (proto = primitive, trophic = food) organisms.

THE SULPHUR ORGANISMS

The sulphur bacteria can live in a strictly chemical manner, requiring no sunlight, chlorophyl, or organic compounds. They obtain their energy by the oxidation of sulphuretted hydrogen to form granules of sulphur. These bodies are deposited in their cells and are then oxidized to form sulphuric acid. The reaction takes place in two phases:

1. $2H_2S + O_2 = 2H_2O + S_2$
2. $S_2 + 2H_2O + 3\ O_2 = 2H_2SO_4$

The visible results of these reactions are seen in the granules of sulphur which are deposited in the bodies of the long thread-like organisms (Fig. 27) and in the sulphuric acid produced. These bacteria are found wherever an abundance of H_2S is being produced for their assimilation, so they are likely to be discovered in the neighborhood of sulphur springs or in stagnant water where the decaying remains of plant life give off an abundance of this gas. A common habitat is along sluggish brooks into which a small quantity of sewage has found its way, particularly in the spring and fall. They form an almost white growth on the decay-

FIG. 27.—Some sulphur bacteria: *A* and *B*, *Thiothrix annulata* Molisch, a thread-like bacterium, showing the habit of growth and an older piece with colorless rings, cells filled with sulphur granules; *C*. *Spirillum granulatum* Molisch, with spheres of sulphur.

ing vegetation along the sides of such streams, other bacteria having destroyed the proteids to produce the hydrogen sulphide for their use. The sulphur organisms appear and disappear in cultures, suggesting some form of resting body, though none was found by Ellis.[5]

The sulphur organisms can be starved by placing them for a day or two in pure water in which there is no hydrogen sulphide, when all the sulphur granules will disappear from their bodies (Fig. 28). The sulphur granules, as reserve food, have exactly the same function as starch or fat in the higher plants. If bacteria that have lost their granules are again placed in water where there is hydrogen sulphide, the sulphur will reappear. The organisms were starved in *B* by depriving them of all H_2S.

An Acid-resistant Sulphur Organism.—Some of the sulphur bacteria do not store sulphur in their bodies. One of the more important of these organisms is *Thiobacillus thiooxidans* studied by Waksman and Joffe.[27] This organism is strictly autotrophic,

being able to derive its energy from the oxidation of sulphur and its carbon from the dissolved carbon dioxide in the soil, soil air containing 0.05 to 6.91 per cent derived from decaying organic matter.[2] Such an organism would not require any carbon compounds to supply it with oxygen, as do many of the sulphur-fixing varieties. This organism has the further distinction that it is able to survive the greatest concentration of acid of any known living organism and can produce acid[26] even in 1 to 5 per cent of sulphuric acid. Growth will cease, however, if the organisms are not supplied with carbon dioxide or oxygen, the former being necessary for the carbon assimilation and the latter for respiration in the form of sulphur oxidation.

It is particularly to be noted that since these sulphur organisms are able to live and thrive in an entire absence of sunlight, they could have lived on the earth before it was habitable even for the green plants.

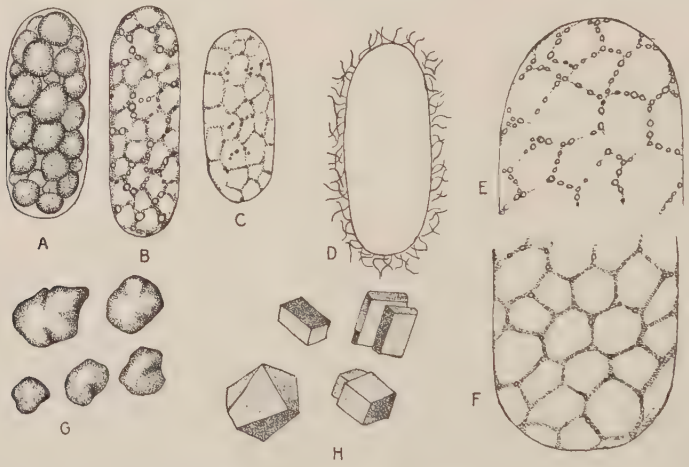

FIG. 28.—Giant sulphur bacterium, *Hillhousia mirabilis: A*, dark sulphur bodies filling the entire cell; *B*, similar specimen kept in tap water for one week, the sulphur globules have been used up in respiration; *C*, specimen kept for a few days in 2 per cent KOH; *D*, cilia fixed in 40 per cent formaldehyde; *E*, specimen treated for 14 days with acidulated pepsin-glycerine to digest the proteins; *F*, end of individual treated for 14 days with concentrated solution of Na_2CO_3, granules dissolved but network remains; *G*, isolated sulphur globules obtained by crushing the cells; *H*, small crystals of sulphur obtained by allowing live organisms to dry up, the sulphur osmosing out of the cells. Figs. *A* to *D*, × 1,000; *E* to *H*, × 2,000. (*After West and Griffiths.*)

The Purple Sulphur Bacteria.—In stagnant waters, bacteria with red coloring matter known as the "purple bacteria" (erythrobacteria) not only utilize the sulphur compounds but have a red pigment, *bacteriopurpurin*,[15,12] mixed with a green pigment, *bacteriocholin*. The red pigment, owing to its color, absorbs the red or heat end of the spectrum even into the infra-red. The products which are evolved by the action of the sunlight on the bacteriopurpurin are not known but starch and oxygen bubbles have been seen.

It would seem that these organisms have two methods for obtaining their food and two chances for obtaining it. In the sunlight, they can use their coloring matter and utilize the CO_2 in the air, while, if forced

to live in the dark, they exist on hydrogen sulphide; but Molisch's experiments would indicate that this theory is not correct. A very dilute protein medium such as 0.5 per cent peptone, or the soluble materials which come from decaying vegetation such as hay, is necessary for growth, and if this type of food is supplied, no other method of nutrition is necessary.

These erythrobacteria are very susceptible to diffuse daylight and are attracted to a moderate illumination but not to intense light. They seem to grow away from other species in mixed cultures, if exposed to a diffused and not too strong light. Other bacteria are indifferent, but the purple species are attracted toward it so that the latter free themselves from competition by their light reaction.

In addition to the colored sulphur bacteria, there also occur, at times, other colored bacteria; short rod- or egg-shaped cells often coat the stones a bright, but dark red, but do not store sulphur. These organisms are also very sensitive to light so it may be assumed that they are able in some way to utilize it.

The Purple Coloring Matter May Be a Primitive Chlorophyl.—The purple bacteria present an interesting group since they may represent the beginnings of the development of a coloring matter capable of utilizing the sun's energy for the fixation of carbon dioxide, and although, according to Molisch's experiments, they are not able to make extensive use of it now, they represent a physical connection between the colorless bacteria and the chlorophyl-bearing types. In the dark they remain quiet; in the light they are in active motion. Molisch suggests that they are able in the light to assimilate organic material, and by these assimilated products they are stimulated in activity. The fixation of carbon dioxide, however, may have arisen in later species as a development of this assimilation of organic products.*

The Energy Released.—The oxidation of hydrogen sulphide to sulphur and then to sulphuric acid would seem to represent a rather inefficient method to obtain energy, but Fischer[8] estimates that, in the first of these processes, the bacteria obtain 0.38 calories from every gram of H_2S converted into sulphur and that the oxidation of a gram of the sulphur will yield 6.5 calories. These high-calory values of the sulphur compounds indicate that the organisms capable of utilizing them are at a decided advantage over others oxidizing carbohydrates.

THE IRON BACTERIA

The iron bacteria were believed by their discoverer, Winogradsky,[31] to have a similar chemical or prototrophic source of energy, arising from the oxidation of iron compounds, but the physiology of these organisms is still in dispute.[4] The grouping of iron bacteria is based by

* The colorless and red sulphur bacteria have been monographed by Bavendamm.[3]

Winogradsky on a physiological function, the oxidation of iron compounds, and includes a variety of morphological forms.[20] The group should be limited[14] to include only the iron bacteria which deposit iron oxide in the layer of slime which surrounds the cells.

These organisms are abundant over the world in water containing iron. Winogradsky's method for obtaining these cultures was very simple. A handful of hay was boiled, cut up, and placed in a tall cylinder, about 50 centimeters high, a little freshly made iron hydroxide being sprinkled over it before it was filled with rain water. After 1 to 2

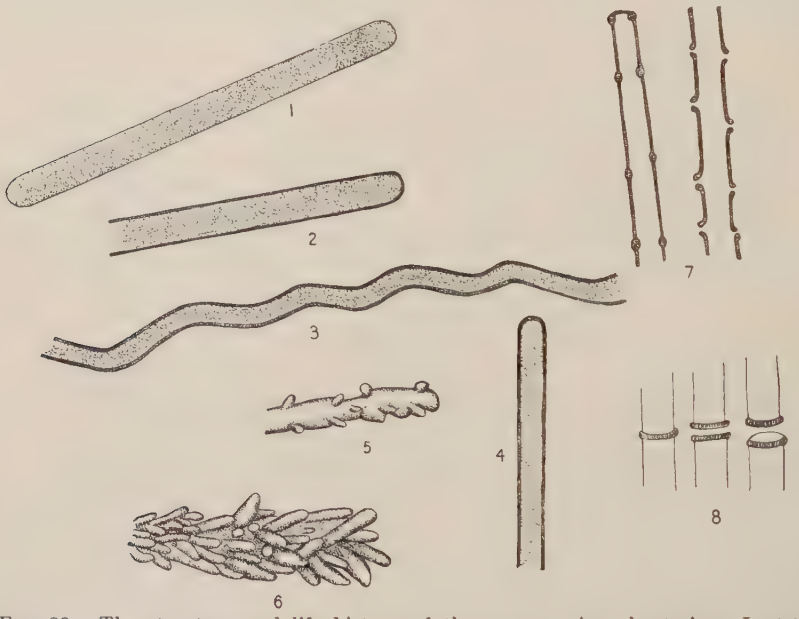

FIG. 29.—The structure and life history of the common iron bacterium, *Leptothrix ochracea:* 1, the young bacterium before the mucilaginous and iron layers have formed; 2, the thickened wall of an older individual containing iron; 3 and 4, old cells; the latter being typical and the former, a somewhat wavy individual; 5, 6, development of the conidia on one of the plants; 7, longitudinal section showing the formation of the wall thickenings preparatory to cell division, and the walls after division has occurred; 8, cell division shown schematically, surface of the cells. (*After Ellis.*)

weeks, yellow-brown spots would appear on the upper surface of the water and on the walls of the vessel, these spots under the microscope being a tangled mass of thread-like bacteria, *Leptothrix ochracea.*

The organisms. *Leptothrix ochracea*, the commonest of iron bacteria, is a large filamentous species with rod-like cells surrounded by a gelatinous sheath in which the hydrated oxide of iron is deposited.

Ellis[5] removed the iron from the sheath and left the cell contents unaltered with no trace of iron inside. The first sign of cell division is the thickening of the walls in spots (Fig. 29). These thickened nodes really are rings around the cells. These rings split and each end of

the new cells has a thickened circular ring. Young cells do not show thickened walls and do not contain any deposition of iron. The threads sometimes show a warty appearance as the conidia which reproduce the organism originate as short protuberances from the side; the branch sometimes assumes a hedgehog-like appearance, occasionally the threads curve as they become old (same figure, 6 and 3).

Numerous other varieties of iron-depositing organisms occur[16,4] with various shaped filaments and methods of reproduction.

Growing These Organisms.—Iron bacteria must be grown in a liquid medium which contains only a very small percentage of inorganic salts. Lieske[13] grew one of the typical forms in the following:

$(NH_4)_2SO_4$	1.5 grams
KCl	0.05 gram
$MgSO_4$	0.05 gram
K_2HPO_4	0.05 gram
$Ca(NO_3)_2$	0.01 gram
H_2O dist	1,000 cubic centimeters

Iron is added in the form of thin shavings to the cultures after they have stood for some days in the open absorbing oxygen and carbon dioxide. If other pure metals are substituted for the iron, the growth will not appear, so that iron would seem to be indispensable for these organisms, although Molisch[16] claims that they will grow without either iron or manganese, such additions only accelerating growth.

The Physiology of the Group.—The group is of importance biologically because of the deposition of iron in the outer sheath and its supposed formation of large deposits of bog iron ore. Both of these phenomena are the basis of controversy.

Winogradsky's original suggestion was that the deposition of iron took place according to the equation:

$$4FeCO_3 + 6H_2O + O_2 = 2Fe_2(OH)_6 + 4CO_2$$

This equation has not been seriously questioned, but the three points which he believed had been proved are still in dispute:

1. The deposition of iron only occurs in water which contains iron in the ferrous condition.

2. Ferrous carbonate is absolutely necessary for the growth of these iron bacteria.

3. Only the living threads contain iron and it is, therefore, the result of the activity of the plant itself.

The first and second of these propositions have been shown by Molisch[14,16] to be without foundation, as the organism will grow in a weak solution of peptone water entirely without iron and with iron-free sheaths. This fact, however, as Lieske[13] points out, does not conclusively prove that the organisms do not derive some benefit from the oxidation of the iron carbonate. If the plant did utilize the iron, some of it should be found in the cells, but a microscopic examination by means of chemical tests for the iron in parts of the plant, other than the wall, have led to negative results.[14] As Molisch points out, everything which enters the cell of the bacterium

must, therefore, pass through this sheath which acts as a filter to retard the passage of iron compounds. These chemicals, held on the outside in such a position that they are readily oxidized, are acted upon by the oxygen dissolved in the water and are deposited there as an insoluble compound bound in the sheath. In the same way, if some compound of the nearly related element, manganese, such as the manganese hydroxide, is placed in the water instead of the iron, the sheath becomes filled with insoluble manganese oxide. In growing these organisms, this same investigator found that they could be continued indefinitely for many generations in water to which no iron had been added. Some similar purely physical process may account for the iron in the walls of the gelatinous sheath of the iron-bearing filaments, since manganese will also be absorbed. Similar cases have been observed in other plants in the selective absorption and deposition of colloidal gold from culture solution.[30] If *P. glaucum* or *O. lactis* is grown in solutions of colloidal gold which contain tannin or gum arabic, the gold is absorbed but retained in the walls even of dead mycelium, the accumulation producing a blue color in the uncuticularized wall. The metal from such colloidal suspensions never penetrates beyond the membrane.[22]

The other viewpoint with regard to the utilization of the iron may seem almost as plausible. The iron, even if outside the plant body in layers around the iron bacteria, may be there as a result of life processes. The oxidation of the ferrous iron, whether in organic or inorganic combination, is not a physical process pure and simple, in which the iron is laid down passively in the outer layers of the organisms, but the organisms absorb it and bring about the changes before its deposition. Only living filaments store it; dead filaments do not have it. Whether the organism derives an energy from this oxidation process is undecided, but since the living plasma membrane regulates the intake and the accumulation, it would seem to serve some purpose in the cell economy.[16]

The two viewpoints on the utilization of iron may be summed up as follows: That the iron is only in the outer gelatinous sheath is admitted by all. One group of investigators holds that it enters the cell through this layer, undergoes oxidation, and the organism derives energy from the process. The other group claims that the deposition is a purely chemical and physical phenomenon similar to that which might take place in any thin layer of jelly, and that oxidation occurs from the outside.

The Formation of Bog Iron Ore.—Large deposits of iron ore, usually of low grade in bog land, have been ascribed to the action of the iron organism.[11] Ellis[5] has described these organisms in the iron deposits of England and Scotland as of the Devonian and Carboniferous periods, although some geologists[25] doubt the existence of plant life before the Carboniferous era.

The bog iron-ore deposits are frequently filled with coccus and thread-like forms and since about 90 per cent of the iron ore of the world comes from the bog and sedimentary ores, their relation to such deposits is of great interest. Harder studied these deposits and the associated organisms in various parts of the world, and believed the work of deposition to be largely the results of the activities of various thread bacteria on iron compounds, such as solutions of ferrous bicarbonate, ferric hydroxide being deposited in the cell walls.[11]

Naumann[20] and others are doubtful as to such a deposition in the iron strata which he investigated, since the common iron bacteria that are now the prevailing type in swamp waters were absent, and a morphologically doubtful species never seen today seemed to be the principal form. This type is a very small coccus, 0.5 to 0.7μ × 1.5 to 2μ.

Other Fossil Bacteria.—Walcott[29] has described bacteria for the pre-Cambrian rocks of this country; Moodie[18] has found spherical forms, which he considered to be Micrococci, in the bony carapaces of fish-like forms in the Devonian; and Renault[23] has evidence of their existence in the Carboniferous. The bacteria can be considered to be among the earliest if not the very earliest inhabitants of the world, in spite of the fact that their small size and delicate structure make them more subject to destruction than the majority of the fossils which have persisted in the rocks.

While bacteria are among the oldest plants on the world, as Walcott has shown by the fossil-bearing rocks of central Montana, these ancient forms were probably harmless, and it is only after the Coal Period that infected wounds in animals were seen. The action of bacteria on the shells of these primitive animals is the first indication of pathogenicity.

OTHER OXIDIZING BACTERIA

Hydrogen-Oxidizing Bacteria.—Another set of interesting oxidizing organisms are those of the group which assimilate hydrogen by oxidation according to the equation:

$$2H_2 + O_2 = 2H_2O$$

The cultures derive their oxygen from the air, but if the air is excluded, they can still oxidize the H_2. If organic substances are supplied along with the hydrogen, they will use both, converting the organic compounds into CO_2 and the H_2 into H_2O. They are apparently able, however, to assimilate CO_2 from the energy derived from the oxidation of the H_2 which must be supplied at the same time.[9,24]

Methane-Oxidizing Bacteria.—Methane (CH_4) is another gas which may be utilized by another series of bacteria. Methane is released in quantity from decaying vegetation especially in swamps and lowland covered, at times, by water. The methane-oxidizing bacteria must be supplied with an abundance of oxygen in order to grow abundantly. Methane is very abundant and these organisms are probably much more common than is generally recognized.[19]

The feature which is common to all of these organisms is the simplicity of their foods and the derivation of energy from their oxidation. This oxidation process is most clearly marked in the sulphur bacteria, although almost as distinct in the hydrogen and methane varieties. The oxidation of iron compounds must still be regarded as a doubtful source of energy.

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CHAPTER VIII

THE NITROGEN CYCLE AND THE FORMATION OF NITRATES

The organisms discussed in the preceding chapter are among the most primitive known, their physiological process for the evolution of energy being the very simplest, although their structure may be complex.

Other forms of microorganisms make use of the organic-nitrogen compounds contained in the soil, oxidizing them and rendering the nitrogen available for higher plants. Two distinct processes are involved:

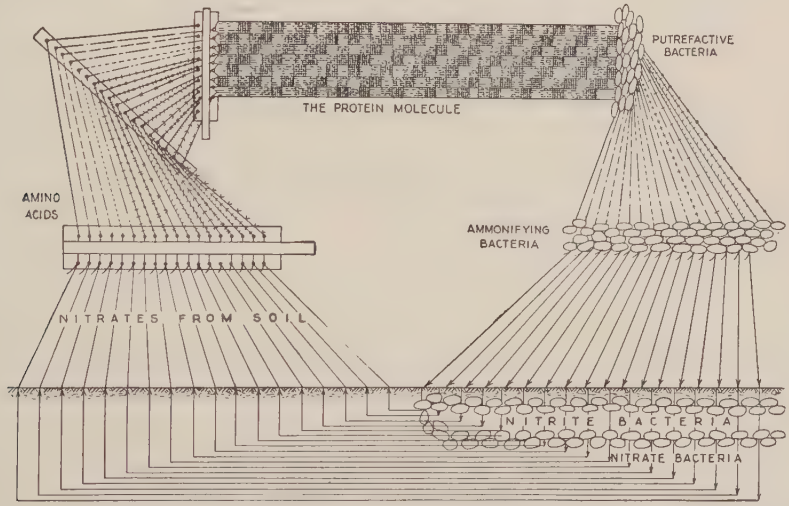


FIG. 30.—The protein molecule, built up from the nitrates of the soil and carbohydrates into amino acids, woven in the living plant or animal into a fabric of complicated pattern, is broken down by the bacteria or other microorganisms into amino acids, ammonium compounds, and finally into nitrites and nitrates.

1. The decay (*decomposition, putrefaction*) of the proteins of plants and animals by a series of organisms with *ammonia* as the principal end product of the nitrogenous constituents.

2. The conversion of this ammonia into nitrites and nitrates.

When leaves fall upon the soil in the autumn, or when an animal is buried in the upper layers of the soil, a series of organisms attack the proteins and destroy them. Special series of organisms change ammonia into nitrates, which may be again built up into the protein molecules of the higher plants. Such a tearing down and building up has

already been shown for carbon and similar ones can be made for sulphur, iron and nitrogen. The nitrogen cycle is shown diagrammatically in Fig. 30.

The destruction of the proteins is the work, not of one, but of a series of organisms known as "ammonifiers;" another series forms nitrates. Each member of such a series uses the products of the one which preceded it, a characteristic of all such changes in nature. One organism carries a reaction to a certain point and then stops, while another organism takes the products and utilizes them for its production of energy. In the oxidation of nitrogen, one organism uses the ammonia products that have been produced by the putrefaction organism, while the second type utilizes the nitrites formed from the ammonia. The cycle of destruction may be a long one. Kostychev found that the leaf mold from oak leaves still contained, after they had been decomposing 12 months, 2.73 per cent of protein nitrogen and only 0.25 per cent of the simpler nitrogenous compounds. The former are valueless to the higher plants until they have been converted into the latter, and the process is apparently very slow. Green plants, as a rule, absorb and utilize nitrate better than ammonium compounds, although it is claimed that some can do so almost as readily.

In order to understand the chemistry of the nitrogen cycle (with its purely chemical series of changes), a short account of the proteins themselves will be necessary.

The Chemistry of the Protein Molecule.—The most complex chemical combinations with which the chemist has to deal are the molecules of the protein compounds. They are so complex that it is only recently that a little insight into their internal constitutions has been gained and all that is known today is still very fragmentary. They contain about 15 to 19 per cent of nitrogen, 52 per cent of carbon, 7 per cent of hydrogen, 23 per cent of oxygen, and 0.5 to 2.0 per cent of sulphur, with occasionally traces of iron and phosphorus. These protein molecules are very loosely united internally so that it is possible to disturb the internal constitution of them without much force from the exterior, that is to say, they are very *labile.* It is in this lability that the weakness and the strength of the protein molecule lie, for, while it can change readily within certain limits to meet shifting conditions, too extreme changes caused by exterior forces wreck the finely built internal structure beyond repair. The proteins are all colloids, although many of them are capable of crystallization, but the colloidal structure may be destroyed by heat, alcohol, or acids.

The Protein Complexes.—The proteins combine in still more complex compounds. Sometimes, the combination is between one or more proteins united with nucleic acid to form the *nucleoproteins,* which are very abundant in tissues where the cell nuclei are very large as compared to the cytoplasm. One of the chief sources of nucleic protein is the

cells of the yeast plant. From the formula for nucleic acid from yeast ($C_{40}H_{59}N_{14}O_{22} \cdot 2P_2O_5$) it will be seen that they are comparatively rich in phosphorus. The nucleoproteins can be hydrolized, *i.e.* split by the action of alkalies into the proteins and nucleic acid.

Protein molecules are very large as a result of the complex combinations and the numerous atoms which compose them. The formula for globin, the protein which is the basis of hemoglobin, is given (Plimmer) as $C_{726}H_{1174}N_{94}S_3O_{214}$. The molecular weight of such a formula would be over 16,000.

The Amino Acids.—The protein molecule is made up of various combinations of the 17 or 18 amino acids and ammonia. These acids are united in different proportions in the various proteins as can be seen from the following table:

	Casein from milk	Oval-bumin from eggs	Gliadin from wheat	Zein from corn	Edestin from cotton	Legumin from peas
Glycocoll	0.00	0.00	0.00	0.00	3.80	0.38
Alanine	1.50	2.22	2.00	13.39	3.60	2.08
Valine	7.20	2.50	3.34	1.88	6.20	?
Leucine	9.35	10.71	6.62	19.55	14.50	8.00
Proline	6.70	3.56	13.22	9.04	4.10	3.22
Oxyproline	0.23	?	?	?	?	?
Phenylalanine	3.20	5.07	2.35	6.55	3.09	3.75
Glutaminic acid	15.55	9.10	43.66	26.17	18.74	13.80
Aspartic acid	1.39	2.20	0.58	1.71	4.50	5.30
Serine	0.50	?	0.13	1.02	0.33	0.53
Tyrosine	4.50	1.77	1.61	3.55	2.13	1.55
Cystine	?	?	0.45	?	1.00	?
Histidine	2.50	1.71	1.49	0.82	2.19	2.42
Arginine	3.81	4.91	2.91	1.55	14.17	10.12
Lysine	5.95	3.76	0.15	0.00	1.65	4.29
Tryptophan, about	1.50	present	1.00	0.00	present	present
Ammonia	1.61	1.34	5.22	3.64	2.28	1.99
Total	65.49	48.85	84.73	88.87	82.28	57.43

The total yield is much below the total percentage to be expected theoretically, due probably to the crude methods of analysis.

Inside the protein molecule, the amino acids are grouped together as *polypeptids* (Fig. 31, AF). One of the most important advances made by protein chemistry came when Emil Fischer was able to produce such polypeptids synthetically by uniting two or more of the amino acids. Biological chemists felt that they were on the way to build a synthetic protein. The synthetic combinations which Fischer made in the laboratory are similar to those which he was able to obtain by the splitting of protein into polypeptids.

Isolating the Amino Acids.—The chemist uses various means to break up the protein molecule into its constituent *amino acids*, the general process being called "hydrolysis." The chemical means are (1) boiling with acids, (2) boiling with alkalies, (3) using various proteoclastic (proteo.= protein, clastic = breaking) enzymes derived from plants and animals. In hydrolyzing them by boiling with concentrated hydrocloric acid, for 6 to 24 hours, the solution becomes a dark brown. The hydrolyzed products are to be found in the solution. A weaker hydrochloric acid, 20 per cent, may be substituted and the mixture boiled longer, 24 to 48 hours. Sulphuric acid of a strength of 25 to 33 per cent or a mixture of sulphuric and hydrochloric acids may be substituted. Alkalies are not generally used, as some of the amino acids are entirely destroyed in the process, although they are very complete hydrolizers. When enzymes such as trypsin and pepsin, protein digesters, are employed, the hydrolysis is never quite complete, and they can not be depended upon for quantitative work.

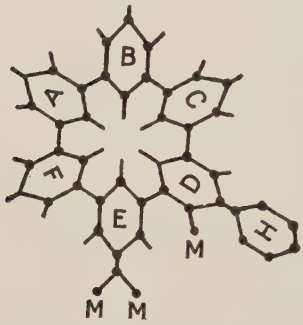


Fig. 31.—Diagram of a protein molecule. (*From Adami.*)

After amino acids are in solution, the problem is to separate them. Tyrosine and cystine are insoluble in neutral aqueous solutions so all that is necessary is to neutralize the acid solution containing them and they will crystallize out. The isolation of the others is more difficult but is effected by using various acids after the tyrosine and cystine are removed by crystallization. Usually two solutions are used, one amino-acid group is precipitated out of one portion and the other group out of the other portion.

The Amino Acids Found in the Protein Molecule.—The chemist finds about 17 or 18 of these acids, in addition to ammonia, in the protein molecule, dividing them into four groups:

Group A:	
1. Glycine or glycocoll	$C_2H_5NO_2$
2. Alanine	$C_3H_7NO_2$
3. Valine	$C_5H_{11}NO_2$
4. Leucine	$C_6H_{13}NO_2$
5. Isoleucine	$C_6H_{13}NO_2$
6. Norleucine	$C_6H_{13}NO_2$
7. Phenylalanine	$C_9H_{11}NO_2$
8. Tyrosine	$C_9H_{11}NO_3$
9. Serine	$C_3H_7NO_3$
10. Cystine	$C_6H_{12}N_2O_4S_2$
Group B:	
11. Aspartic acid	$C_4H_7NO_4$
12. Glutamic or glutaminic acid	$C_5H_{14}N_2O_2$
Group C:	
13. Arginine	$C_6H_{14}N_4O_2$
14. Lysine	$C_6H_{14}N_2O_2$
Group D:	
15. Histidine	$C_6H_9N_3O_2$
16. Proline	$C_5H_9NO_2$
17. Oxyproline	$C_5H_9NO_3$
18. Tryptophan	$C_{11}H_{12}N_2O_2$
19. Ammonia	NH_3

THE DESTRUCTION OF THE PROTEINS BY MICROORGANISMS

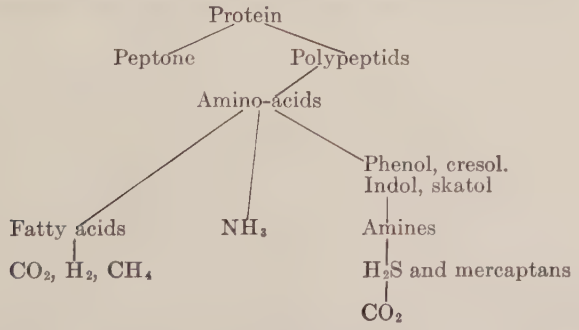
The destruction of the proteins, the reverse of the syntheses, is largely done by bacteria and molds, but, even in their absence, the action of the enzymes present in the dead cells themselves and the oxidation of the air assist to some extent. The greater part of the destruction, however, comes from the attacks of bacteria and molds, a large number of which are able to secrete proteoclastic enzymes to break the proteins into simpler products.

Cohn[2] found bacteria in putrefying liquids, assumed them to be all one variety, and named them *Bacterium termo*, although Pasteur[8] had already shown that at least two sorts of organisms were involved, since the putrefaction occurs both in the presence of air and in its total absence. This organism which destroyed protein in the absence of air, he had called *Vibrion butyrique*, since one of its products was butyric acid. This early work was all done in the absence of pure cultures and it remained for Hauser,[4] using the plating-out method, first to give an intelligent picture of some of the organisms involved. Since the principal organism seems to appear under many forms, it received the generic name "Proteus," and, since it was widespread, the species name of "vulgaris." This same organism is now called *Bacillus proteus vulgaris*, *Bacillus vulgaris*, or *Bacterium vulgare*.

While other bacteria besides this species perform the same work, this is the commonest, especially in putrefying flesh. A widespread species, once frequently occurring in wounds which would not heal normally, excretes a green color and digests the adjacent cells of the tissues. This organism is known now as *Bacillus pyocyaneus*. It is closely related to the soil organism which also destroys proteins and produces a similar green color, *Bacillus fluorescens liquefaciens*.

The action of the bacteria is like that of acids and alkalies on the protein molecules in that they split them up into albumoses and peptones (of which we know very little chemically) and into the still simpler amino acids.

The Products of Bacterial Action.—The following general scheme for the destruction of the proteins (from Rippel[9]) indicates some of the intermediate steps and the more important end products:



This destruction has been followed in the fibrin of lean meat and in the casein of milk. Very little is known about the destruction of the proteins to the point where the amino acids are formed, but after this stage is reached we know many of the putrefactive products. The

amino acid which seems to have received the most attention is *tryptophan*, a common constituent of proteins, whose decomposition by putrefactive bacteria gives the two cleavage products, *indol* and *skatol*, aromatics associated with the bad odors of putrefaction. Phenol and cresol, other intermediate products, have been found in feces. Indol and skatol have been particularly studied because they are found in the intestine as the result of alimentary putrefaction by the intestinal organisms. Attempts have been made to estimate the amounts of phenol and indol produced by putrefaction, but it is doubtful if it is possible to do so accurately.

Other amino acids found in putrefaction products are glycocoll, leucine, isoleucine, arginine, lysine, histidine, as well as a number which come from these amino acids, such as formic, acetic, propionic, butyric, oxalic, and succinic acids.

The gases of putrefaction had been studied before 1880, but with doubtful results in the light of more recent studies.[5,7] The gases which are produced are carbon dioxide, hydrogen, and ammonia. Combinations of the latter are interesting from the standpoint of the nitrogen cycle and of continued soil fertility, since a soil is most likely to become impoverished in nitrogen compounds. The ammonium compounds, formed in the rotting of manure and particularly in the fermentation of urine, escape into the air unless they are combined with some chemical which will bind them so that they may be returned to the soil for further transformation for the use of the higher plants.

THE SCHLÖSING AND MÜNTZ EXPERIMENTS

The same changes which occur in nitrogenous compounds in the soil take place also in sewage, and, in fact, were first studied in sewage by two French chemists, Schlösing and Müntz in 1877. These investigators took a glass tube of large bore and a meter long, filled it with a mixture of sand and lime, and allowed sewage water containing ammonia to trickle slowly through it. After a few days, the water which came through contained nitrate, while the ammonia which had been in the sewage water was found to be oxidized. When they passed chloroform vapor through the tube while the water was percolating, however, the nitrification did not occur. The chloroform had stopped the vital activities of the bacteria and the ammonia passed through the sand and lime, unchanged. Chloroform acts only on living organisms and has no effect on a chemical reaction, so they concluded, correctly, that *the chemical change from ammonia to nitrates was induced by living organisms*. The ammonia was the food which the sewage bacteria oxidized to form nitrates.

This work of Schlösing and Müntz is the basis of our knowledge of the processes which occur in the destruction of ammonia, not only in sewage

but in soil and in every rotting pile of manure. From an economic standpoint it was one of the most important chemical discoveries of the century, for it gave a clew to soil fertility in its relation to the nitrates. The whole process, from ammonia to nitrates, took place in a long tube in their laboratory and no attempt was made to separate the various series of organisms which played a rôle. As in so many changes which occur in nature, one set of organisms makes use of the by-products of another, the reactions being linked together and one dependent on the other.

The isolation of the organisms which oxidized the ammonia into nitrites and the nitrites into nitrates was a difficult feat and was not accomplished until many years later.

FIG. 32.—S. Winogradsky (1856–). About the time he was isolating the nitrogen organisms from the soil.

THE NITRITE FORMERS

Investigators worked for years attempting to isolate the organisms in pure cultures where the reactions could be studied, but all attempts were futile until a brilliant Russian scientist, Winogradsky[10] (Fig. 32), found the method, his contribution being, like that of Schlösing and Müntz, another "path-breaking" discovery. The secret lay in the use of media which contained no peptone, proteins, or sugars. In fact, the presence of any organic substance in the media seemed to prevent their growth. Gelatin was being used at this time as the basis of many of the media, with meat juice and peptonized meat added, so, no nitrifying bacteria would grow. Winogradsky suspected the cause of the trouble and made his medium of inorganic salts held in a silicate jelly known chemically as potassium silicate or "water glass." His solution contained the following:[13]

Ammonium sulphate $(NH_4)_2SO_4$	2 grams
Magnesium sulphate $(MgSO_4)$	0.5 gram
Dibasic potassium phosphate (K_2HPO_4)	1 gram
Calcium chloride $(CaCl_2)$	trace
$MgCO_3$	in excess.
Distilled water	1,000 cubic centimeters

It will be particularly noted that the nitrogen was supplied in the form of an *ammonium compound.*

The water glass furnished a clear translucent jelly in which bacterial colonies could readily be observed.

The preparation of the water glass was carried on with the greatest care. Water glass (with a specific gravity of 1.05 to 1.06) is mixed with hydrochloric acid (with a specific gravity of 1.10) by pouring the water-glass solution into the acid and not the reverse. The combination is then dialyzed through a parchment paper membrane to remove the acid. The water glass itself must be colorless and clear, and dialysis is complete when the water from the dialyzing membrane gives no precipitate with silver nitrate. The solution should be clear and not opalescent, should contain about 2 per cent silicic acid, should keep about 3 months, and should stand sterilization at 115 to 120°C. But Winogradsky's difficulties were not yet at an end. He used the usual dilution and plating-out method but could find no colonies of the nitrite organisms, although he used all precautions. But when no colonies appeared after a long time, he put them under the compound microscope, and then the reason for their absence

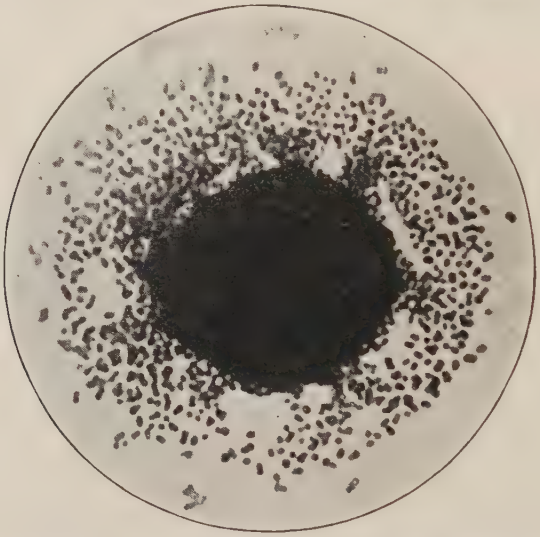


FIG. 33.—Surface colonies of Nitrosomonas on silicic acid gel, stained with carbol-fuchsin without removing the gel, × 250. (*From Gibbs.*)

was evident; these nitrifying bacteria never form colonies of size visible to the naked eye. After 2 weeks' growth, they appear as little granules in the solid media.

Even under the microscope they were visible only after they had been incubated for 2 to 6 weeks. The growth could be detected by chemical means before the colonies were visible under the microscope. Nothing could be gained in examining the plates until they gave a pronounced nitrite reaction, *i.e.* if a little of the jelly-like mixture was cut out and placed in iodine-starch solution or in diphenylamine, a deep blue color resulted. The difficulty was so great in obtaining growths on a solid medium that Winogradsky always grew them for some time in the same chemicals in water, *an enrichment culture*, before putting a little of this liquid into the solid silicate jelly. A little soil was added to the liquid culture solution, soil being obtained from all quarters of the world in order to compare the bacteria which would appear. Two types of the nitrite-forming organism (Nitrosomonas) were found in the liquid enrichment cultures. In one form, the growth was in a zooglea (a jelly-like mass of bacteria); in the other, it was single cells, very motile, which made the media turbid.

The colonies of nitrite formers (Nitrosomonas) on silicic-acid gel appear as small refractive bodies, even when magnified 100 diameters (the usual low-power magnifica-

tion), with a main nucleus-like center surrounded by a less dense growth of single cells (Fig. 33).

These organisms oxidize the ammonium sulphate in the medium into a nitrite and derive their energy from the process.

The Organisms.—The nitrite bacteria seem to vary in different parts of the world but are always coccus-like in shape and about 1 micron in diameter in the varieties from European soil, while in soil obtained from Java, they are only 0.5 to 0.6μ in diameter. On the other hand, varieties from the soil of Quito were 1.2 to 1.7μ in diameter, and those from the campañas of Brazil still larger, measuring up to 2μ (Fig. 34). All of these measurements suggest that there may be a number of different varieties, all alike physiologically but variable in morphology, and further suggest the possibility that very different and unrelated organisms may have acquired the habit of

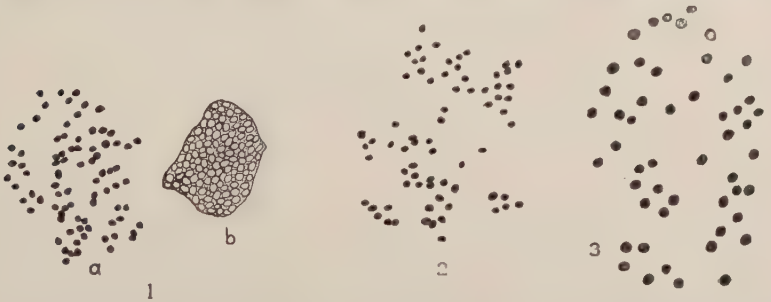


FIG. 34.—Nitrite-forming bacteria from various parts of the world: 1*a*, from Zurich; 1*b*, similar organisms held together by their slime; 2, from Java; 3, from Quito. × 900. (*After Winogradsky.*)

destroying ammonium compounds to form nitrites. The fact that they perform the same chemical work does not necessarily mean that they are related.

Winogradsky called these organisms *Nitromonas* but later changed the name to *Nitrosomonas*. The difference was so great in the group that the organisms were not only given different names, *N. europea* and *N. javaensis* for the old-world forms, but the new-world types were made into a new genus, *Nitrosococcus*.

Beijerinck[1] secured similar results by using agar that had been washed for 2 weeks by simply letting the agar remain in the water until it was freed from the decaying organic material in the agar shreds. Even with this precaution the growth is poorer than on silicate jelly, the colonies require from 3 weeks to 1 month to grow, and the medium is more likely to be contaminated by other bacteria.

THE NITRATE FORMERS

The nitrate formers were about as difficult to grow in pure cultures. The essential difference in the culture methods was the use of a nitrite instead of an ammonium compound as a source of nitrogen, but silicate jelly was found preferable to agar even after a thorough washing. From a rich culture of nitrate formers in liquid media, transfers were made to solid media of the same chemical composition where colonies were obtained and isolated. They were first discovered by Winogradsky in 1891 in soil from Quito which he had been using for nitrite isolations. They were isolated on a water-glass medium but with

nitrite instead of ammonium compounds. Later, washed agar was employed as a thickening colloid.[11–13]

The nitrite solution used was as follows:

Sodium nitrite	1.0 gram
Potassium phosphate	0.5 gram
Magnesium sulphate	0.3 gram
Sodium carbonate, anhydrous	1.0 gram
Sodium chloride	0.5 gram
Ferric sulphate	0.4 gram
Distilled water	1,000 cubic centimeters

With agar, the following combination was found to be successful:

Sodium nitrite	2.0 grams
Sodium carbonate (anhydrous)	1.0 gram
Potassium phosphate	trace
Agar	15 grams
Tap water	1,000 cubic centimeters

Colonies of nitrate formers (Nitrobacter) are slightly more rapid in growth so that they could be seen on the silicate jelly after about 10 days under a 150 to 200 magnification.

Growth on agar plates is extremely slow and even after 1 week nothing can be seen, but, at the end of 2 weeks, little refractive granules can be detected under the magnification of 150 to 200 diameters. The general procedure was to stroke, with the platinum loop, the surface of plates of the above composition with bacteria from a

Fig. 35.—Two nitrate formers: *A*, from Leningrad; *B*, from Quito. × about 2,000. (*After Winogradsky.*)

liquid culture that was rapidly forming nitrates. The growth of the bacteria sought is very slow and the colonies small, so it must be carefully watched or other organisms which also grow on this medium will be mistaken for the true nitrate formers. Winogradsky states that the only way to be sure is to take the small slow-growing colonies and test them out by inoculation into a flask containing the liquid media to see if the growth is continued. They are the only organisms which will grow on such a combination, so if the liquid becomes turbid, they are certainly a nitrate formers.

The organisms are short rods, about 1 micron in length and 0.3 to 0.4 micron in breadth, with pointed colorless ends and a deeply stained middle (Fig. 35). They seem, at times, to have a thin, slimy capsule which does not stain.

The Physiology of the Nitrite and Nitrate Formers.—The interesting mode of life of these organisms must be again noted. These organisms derive their energy from the oxidation of the nitrogen compounds and with this energy are able to fix the CO_2 of the air and to build up the carbohydrates and proteins from it. This CO_2 must be absorbed from the air held in the soil, so that a constant and abundant supply of air must be in contact with them. Such conditions are best supplied by a well-aerated soil, such as would result from careful and thorough cultivation, the soil particles alternating with pockets of air.

Later Work on the Nitrite and Nitrate Bacteria.—These classical experiments of Winogradsky in 1890 have been repeated in various forms by other investigators. Gibbs[3] isolated from soil *Nitrosomonas* and *Nitrobacter*, both on the washed agar plates and also on the silicate jelly plates, making liquid enrichment cultures in which the colonies showed as fine growths under the microscope. Picking up the colonies with a very fine capillary glass pipette, he transferred them to the special nutrient solution. This solution might be a liquid medium of the same type or it might be silicate agar or washed agar. The organisms will continue to grow indefinitely in the liquid medium and still show activity. Absolutely pure cultures of either of these organisms cannot be secured by simply transferring them from one liquid culture to another, although the relative numbers of these types may be greatly increased. Gibbs transferred them fifty-two times and found that there was no diminution in the activity of the organisms even after all this long growth under artificial conditions.

Only 10 to 20 per cent of the colonies isolated from solid media oxidize nitrates when transferred to liquid media. Gibbs is of the opinion that the organisms had been weakened by the growth on the solid medium, but there remains the possibility that, like all organisms, only a small percentage show the characteristics supposed to be common to all. The nitrogen-fixing organisms are very sensitive to light and will not grow nor fix nitrogen unless protected from the light, being very readily killed by direct sunlight. They will stand long periods (in some cases 10 months in the laboratory) without transfer.

The Destruction of Urea.—The destruction of urea is a special form of nitrogen oxidation by a series of bacteria which occur in the urine of most of the warm-blooded animals. The urine, in the process, changes from an acid reaction, such as it has in the body, to a distinct alkaline one, as will be seen from the following reaction:

$$\underset{\text{Urea}}{(NH_2)_2CO} + 2H_2O = \underset{\text{Ammonium carbonate}}{(NH_4)_2CO_3}$$

The process, it will be observed, is a simple hydrolytic one. The organism splits up the urea by the action of an enzyme, *urease*, but the

same hydrolysis of urea can be brought about by adding very basic chemicals or by heating the urea to a temperature of at least 140°C., since urea is a very labile organic compound.

A medium containing only urea and mineral salts will not permit the growth of these organisms; they require, in addition, some substances containing carbon in one of the higher forms in order to build up their cell structures. Fermentation of urea will supply the energy, but some carbon compound must be present to furnish material for the cell itself.

The organisms which bring about this change in urea are either spherical organisms arranged in packets, or long rods, many of which contain spores.

The urea organisms are distributed very widely in nature, being found in air, water, and soil. Miquel tested 104 cases of urea fermentation which had been produced by the organisms from the air and found that 71 of them contained Urococcus and 19 Urobacillus; while 10 were fermented by Eumycetes which also have the power to ferment urea. Of 1,000 bacteria isolated from springs and flowing water near Paris, 15 were urea destroyers; from 1,000 in sewage, 52 were urea fermenters; and in 1,000 from closets, 66 urea fermenters. In dung, about 10 per cent are of this type, so it is not at all surprising that urea breaks down so easily in nature.

Löhnis and Fred[6] suggest that such organisms do not exist as distinct species but are only varieties of the common *B. proteus, coli, prodigiosus, fluorescens,* and *erythogenes.* They note further that the ferment, *urease,* is not confined to bacteria or fungi, but that it is also very abundant in the soy bean.

The nitrogen cycle and the formation of nitrates is complex and the more important facts may be summed up as follows:

1. Two distinct processes are involved:
   a. Ammonification of the protein (decay).
   b. Nitrification of the ammonium compounds.
2. The protein molecule is made up of amino acids combined into elaborate and complex compounds. The chemist divides the 17 to 18 amino acids into four groups. These amino acids are the bricks out of which the various proteins are built.
3. The destruction of the proteins is effected by bacteria and molds.
4. The destruction of protein may be either with oxygen or in the absence of oxygen.
5. The gases produced are carbon dioxide, hydrogen, and ammonia, with methane and hydrogen sulphide at times.
6. The transformation of the ammonium compounds into nitrates was first studied by Schlösing and Müntz in sewage in 1877.
7. The nitrate formers were first isolated into pure cultures by Winogradsky by the use of silicate agar in 1890.
8. The nitrate formers were isolated very shortly afterwards on a similar medium.
9. Urea is destroyed by special bacteria or special strains of bacteria breaking this chemical into ammonium carbonate.

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CHAPTER IX

A GENERAL DESCRIPTION OF THE FUNGI

The fungi are saprophytic or parasitic plants of the Thallophyta group. They are all devoid of chlorophyl but may be colored black, green, blue-green, brown, or yellow, from substances in the cell walls or in the plant body. If no such substance is present, they are white.

The Structure of the Plant.—The vegetative part of the plant or *mycelium* is usually made up of strands or filaments, *hyphæ*. The hyphæ may be long tubes without cross-walls (non-septate) or the tube may be cut up by cross-walls (septate). In numerous species, each cell is provided with many nuclei (coenocytic) although in others, each cell contains but one or two nuclei. The mycelium branches freely and spreads in all directions over the substance (substratum) upon which it is growing.

The mycelium arises from a reproductive body known as a *spore*, the first hypha is the *germ tube*. The wall of the spore and of the germ tube is continuous. The material (food and fungus protoplasm) contained in the spore flows out into the germ tube. This material is usually increased in mass by the presence of numerous large vacuoles. The food is scattered throughout the protoplasm as small refractive granules.

The cell walls of the hyphæ are composed of a special type of fungus cellulose.

Reproduction in the fungi is by spores, asexual and sexual. Both types of spores vary in the manner of production in the different groups and give the systematist an opportunity to arrange the organisms into a scheme of classification. Classification of fungi is largely based on the method of production and shape of the spores.

The asexual spores may be enclosed in a wall forming a sporangium and only set free by the rupture of the sporangial membrane, or, they may result from the budding and pinching off of cells from a hypha, the spores being known then as *conidia*. These spores may be enclosed in a flask-shaped receptacle known as a *pycnidium* or they may be developed free as single stalks or in groups. The fungi sometimes develop cells with thickened walls among the other vegetative cells of the hypha, the *chlamydospores*. The special group of the fungi known as "yeasts" bud off smaller bodies which are released from the parent plant as soon as they have attained approximately the same size.

Sexual spores are produced internally in a sac known as an *ascus*. The *basidiospores* are borne externally on a cell or a row of cells, the *basidium*.

The spores vary in size and in their cell walls. Some have thin, clear membranes while others have thick, brown, sculptured walls. The contents of the spores often include droplets of oil and very small vacuoles. Some of the spores (zoospores) are, for a time, motile by the action of flagella.

The growth of the fungi in nature and in artificial cultures is limited by the nature of the chemicals in the medium upon which they are growing, by temperature, excretion products, light, and acidity.

In general, growth is best in artificial media of a moderate acidity containing some of the ordinary sugars such as dextrose, sucrose or levulose. The rate of growth is best at temperatures of 25 to 30°C., although some grow best at lower and some at higher temperatures. The excretion products of an organism check and finally prevent its growth. Their removal usually means renewed activity in absorption and growth.

Fungus Enzymes.—The fungi dissolve insoluble organic material and render it, by the secretion of enzymes, absorbable. These enzymes are used to dissolve various carbohydrates, such as cellulose, lignin, starch, and various sugars and proteins. Each enzyme is specific, *i.e.* dissolves only one substance. The organic substances, such as suberin and cutin, are impregnated with fatty compounds and are very difficult to dissolve by the action of enzymes.

Very few fungi are parasitic on man or animals, but they are the common producers of abnormal conditions in higher plants. The majority of them are saprophytic (live only on dead material from animals and plants) but the parasitic varieties find their habitat principally on plants. Their parasitism is principally attained by the withdrawal of soluble materials from the host to which they have attached, by clogging the water conducting systems, and by poisoning the adjacent host cells by their excretion products rather than by formation of specific toxins.

The classification of the fungi is based primarily on the morphology of the mycelium and the form and method of origin of the two types of spores.

The following table will show the probable relative positions and classification of some of the common fungi:

THE PRINCIPAL CLASSES OF FUNGI

- Fungi
  - Mycelium usually non-septate
    - Phycomycetes
      - Oomycetes, sexual reproduction by zoospores — Water molds
      - Zygomycetes, sexual reproduction by zygospores — Black mold on bread
    - 1. Ascomycetes (Ascospores)
      - 1. Plectomycetes (no opening in the ascocarp). Penicillium, Aspergillus
      - 2. Discomycetes (ascocarp wide open) — Cup fungi
      - 3. Pyrenomycetes (ascocarp open by a small hole when ripe) — Mildews
  - Mycelium septate
    - 2. Fungi Imperfecti (only asexual spores known)
    - 3. Basidiomycetes (reproduction by basidiospores)
      - 1. Basidiospores, indefinite in number — Smuts
      - 2. Basidiospores, usually four — Rusts, Mushrooms, Puff Balls

The preceding table does not include a number of the minor forms but the more important groups (especially the position of the molds) are indicated. The most important groups in numbers are the Ascomycetes and the Fungi Imperfecti. Both are very diverse in the types and methods of spore formation. The majority of the Fungi Imperfecti will probably eventually find their place among the Ascomycetes but the ascus stage of each has not yet been discovered and described. The name *Fungi Imperfecti* implies, therefore, in the majority of cases, that the full knowledge of the life history is lacking rather than that the fungus is incomplete in all its stages. Some of the *Fungi Imperfecti*, however, may have lost some of the spore forms in the course of their development. The yeasts are Ascomycetes which have lost all the spore forms except the ascus, since any cell seems to be capable of dividing internally into ascospores.

THE TYPE ORGANISMS DESCRIBED

A series of molds, yeasts, and bacteria, carefully selected because of their historical and biological importance will now be described. The external morphology, growth, internal structure, and physiology are given in considerable detail; in some cases, the facts are necessarily

repeated, but each type exhibits some essential facts in the life and growth of microorganisms.

The molds have been used as the beginning types, not necessarily because they are the oldest or the simplest but for the reason that they are large enough to be seen without difficulty by the help of the ordinary microscope. Some can be seen, too, with the naked eye and are, for this reason, more tangible than the very small and simple bacteria. The logical method would be to begin on the bacteria but practical experience has indicated that the reverse order is better from the laboratory point of view.

CHAPTER X

RHIZOPUS NIGRICANS

(THE BLACK MOLD OF BREAD)

One of the common molds of the group *Phycomycetes* (phyco = alga, mycetes = fungi) is *Rhizopus nigricans* (rhizo = root, pus = foot; nigricans = black) the black mold of bread, a large coarse form with strands like fine cobwebs and little white and black balls scattered throughout the mass of the mold growth (Fig. 36).

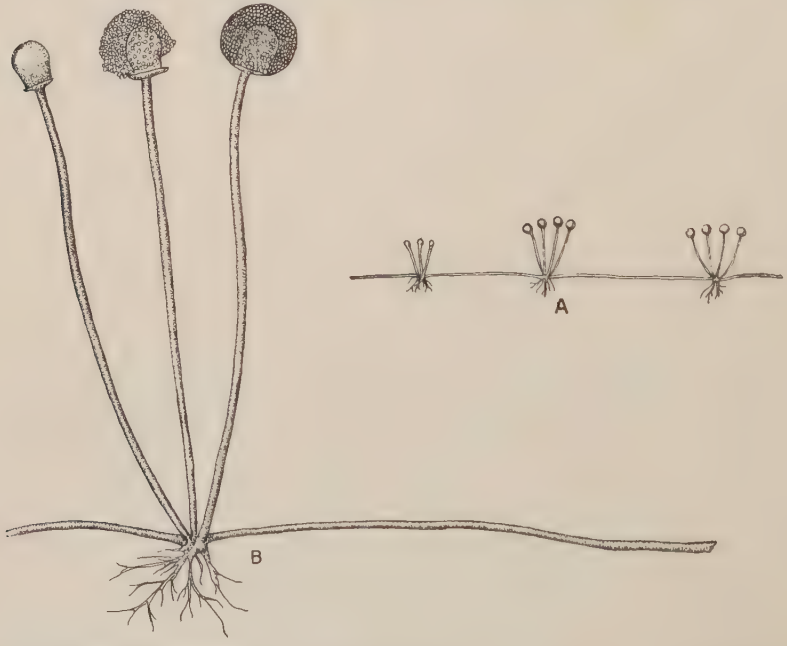

FIG. 36.—*Rhizopus nigricans: A*, growth by runners; *B*, sporangia, sporangiophores, and absorbing hyphæ. (*After von Tafel.*)

The Structure of the Plant.—The body of the mold plant is the *mycelium* (myco = fungus, lium = nail or wart); each strand a *hypha* (hypha = a web, plural *hyphæ*). The small black spheres containing the *spores*, the reproductive bodies of the fungus, are called either *sporangia* or *sporanges* (spore = seed, angium = vessel). The sporangia arise in clusters of three or four from a common base. The upright hyphæ bearing the sporangia, the *sporangiophores* (phore = bearer) have brown, thick, strong walls; the common attachment of the sporangiophores being continued down into the substratum upon which the fungus is growing as coarse, branching, brown, thread-like growths, forms the *rhizoids* (rhiz = root, oid = like) serving the same purpose as roots, absorption and attachment.

The Reproductive Organs.—The sporanges vary greatly in appearance since some contain spores forming, others mature spores, and from many, the spores have fallen away leaving a smooth brownish oval head. If a specimen still containing the spores is selected, the upright column of the sporangiophore will be found to be continued up into the spore mass as the columella (little column) the shape of this organ being variable but constant for each species. After the spores escape from the sporange, the columella is left as the ending of the sporangiophore and part of the wall of the sporange is left as a *collar*. The spore mass can often be seen with the spores still enclosed in the sporange wall but separated from the columella.

The white sporangia are the young ones, the very youngest of all being club-shaped structures. The older black ones containing the matured spores have grown into almost perfect spheres.

The Internal Development of the Sporangia.—It is impossible to follow the growth of the spores from the outside; all that is known of the finer details has been made out by means of sections cut thin enough to be transparent and then stained to differentiate the spores with their nuclei. In such sections, the mycelium contains many small nuclei scattered through the cytoplasm, in contrast to the majority of plant and animal cells where a single nucleus is sufficient for each cell (Fig. 37*A*). Such a cell is called a "coenocyte" (coeno = common or general, cyto = cell). The material in the coenocyte flows up through the sporangiophore into the knob-like end, carrying along both cytoplasm and nuclei in its stream (Fig. 37*B*), to the end of one of the upright hyphæ. The denser portion gathers under the sporangial wall on the outside and later a row of vacuoles cuts off this outer denser portion (Fig. 37*C*) from the inner portion with a more vacuolar cytoplasm and fewer nuclei. This row of vacuoles may be considered as bubbles which fuse to form a single long bubble with the spore material all on the outside. The columella is deposited inside the flattened vacuoles as a distinct wall, *D*. The denser outer portion now cuts up into spores by a process of *progressive cleavage* of the mass from the outside. The process seems to be one of drying out of the spore mass. At first homogeneous, as it dries from the outside, little cracks split it up into smaller fragments containing one or several nuclei surrounded by a layer of cytoplasm. These fragments are at first irregular in shape, *E*, but dry, round up, and become wrinkled to form the spores. The swelling of these rounded spores to form hexagons again fills all the space between the sporangial and columella walls.[24]

The process of spore formation, therefore, is to be considered as an automatic splitting up and drying of bits of the mold protoplasm to spread the species or to preserve them over periods of lack of food and water. It is strictly an *asexual process* comparable to the use of cuttings in the higher plants to secure an increase in number, although cuttings are made up of large groups of cells from leaves or stems.

Germination of the Spores.—Spores germinate in pure water simply by imbibing it, as does a dry seed before a young plant pushes out. The vacuoles inside the spore swell as they become filled with water, increasing many fold the size of the spore itself. If the solution contains no food, however, there is no real increase in the quantity of material, only a dilution of material already stored; the spore is a very concentrated mass of protoplasm and can imbibe much water, but if there are nutrient salts and sugars in the absorbed solution, there is real growth both in bulk and in material. The spore first swells to a large ball and a *germ tube* is put out of one side or sometimes from two sides. This tube increases in length as the food is absorbed and assimilated and finally branches and rebranches to form the mycelium.

The Growth of the Hyphæ.—The hyphæ are granular in appearance from the small particles of food stored in them but are also highly vacuolar since the fungus attempts to build as much in bulk with the limited supply of materials at its command. The first attempt is to reach a new source of food and water and this can be done only

by *growing* toward it. If it is the spore stage, the wind will carry the fungus and spread it; but, after germination has occurred, it is helpless unless it can grow *into* a region where new supplies are to be obtained.

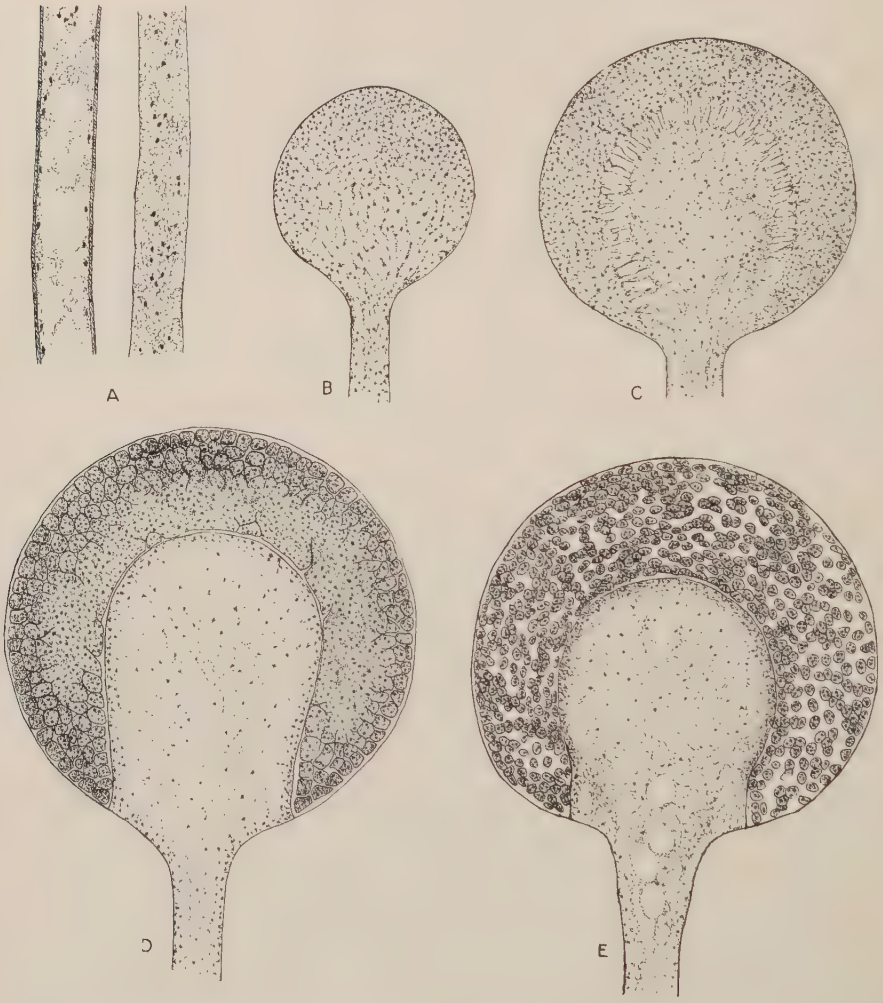

Fig. 37.—Cytology of spore formation in sporangium of *R. nigricans: A*, mycelium stained to show nuclei; *B*, streaming of cytoplasm upwards into young sporangium; *C*, cutting out of spore-forming layer by the formation of a series of vacuoles to form the columella; *D*, columella formation completed and dense outer mass of cytoplasm cutting up into small bits to form spores; *E*, the spores are not yet rounded up. (*After Swingle.*)

In young growing tips, a streaming of the granules in the protoplasm may be seen occasionally (Fig. 38), apparently induced by a sudden change in the environment.[1,2] Rapid transpiration, a transfer to a strong sugar solution, or a sudden change in temperature will start it up. On the other hand, injury will serve as a shock and will stop it.

The growth of the mycelium continues until the formation of sporangia sets in to check it. The question arises: Has the fungus accumulated so many waste products that it attempts to free itself from them by spore formation, or, is it impossible to form spores until a certain amount of reserve protein and carbohydrates have accumulated to feed the spores? The latter is probably correct, but these are questions that are to be met in every plant as it comes to the flowering or reproductive stage.

Very abnormal structures are produced in the hyphæ of some of the Mucors when they are grown in unusual culture media, but if the cells are returned to a normal one, the cells will regain their usual size and arrangement so that the structure of the fungus is not modified permanently by placing in unfavorable surroundings.

FIG. 38.—Movement of protoplasm in germ tubes and branching hypha of *Mucor stolonifer;* movement indicated by arrows; the vacuoles elongate in the direction of flow. (*After Andrews.*)

The Chemical Composition of the Cell Wall.—The cell wall is colorless, composed principally of cellulose but strongly impregnated with a nitrogen-containing material resembling the chitin of animal tissues.

The cell walls of the fungi were early recognized to be different chemically from those of the higher plants and described by de Bary[4] as composed of *fungus cellulose.* This name implies that its base is a cellulose compound and most of the investigators agree with this view point; Richter,[22] for example, says that ordinary cellulose is mixed with other products and very difficult to separate. Mangin[15] found cellulose in a number of fungi but in others a substance which he called "callose," identical with a similar material present in the sieve tubes of the flowering plants. In some fungi, callose is accompanied by pectin; in others, by true cellulose. Callose is to be distinguished by the color reaction (blue) which it gives with a special stain, known as "Bayer's blue."

Gilson[11] was the first observer to note that the cell walls of the fungi contain the same substance as that which occurs in the walls of the horn and nail cells of animals, *chitin.* He demonstrated chitin in fungi, converting it through heating with potassium hydroxide solution at a temperature of 180°C., into another substance,

mycosin, which colors reddish violet in a solution of iodine-potassium iodide containing a trace of acid. The strong alkali had to be removed by repeated washing, so that much of the substance for which the test was to be made was dissolved and lost.

Van Wisselingh,[26] using the same method but a temperature of 160°C. and washing out all of the concentrated alkali with 90 per cent alcohol instead of water, was able to examine sections or the mycelium of fungi without the walls' going to pieces in the washing process, as often happens with water. The color of the walls varied from a reddish violet to a dull brown, being often masked somewhat by the color of untreated walls. Mycosin and cellulose can be distinguished by the color which appears after treatment with alkali and iodine; mycosin is colored reddish violet while cellulose becomes a faint blue. The mycosin color is permanent while the blue can be readily removed by washing with distilled water. A test can be made on the same material which has been used for the mycosin reaction, by adding 76 per cent sulphuric acid, when the violet color will disappear and the blue color due to the cellulose show. Using the above procedure, Van Wisselingh was able to demonstrate chitin in the walls

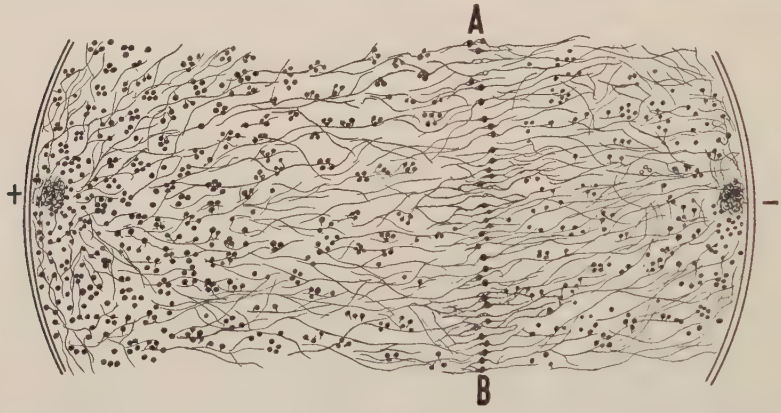

FIG. 39.—Formation of zygospores in *R. nigricans* from *plus* and *minus* strains inoculated on two sides of Petri dish, the zygospores forming near center along line *AB*.

of all the fungi which he tested except those of two groups; facts confirmed later by Wettstein.[28] Many of them contained, in addition, a small or large amount of cellulose.

The cell wall of *Rhizopus nigricans* contains much chitin, the mycelium, the sporangiophore, the walls of the sporangium, and the walls of the spores themselves, all containing it.

Development of Sexual Spores.—Cultures kept in laboratories in various parts of the world developed another type of spore at times. This spore arises from the fusion of the two ends of two hyphæ and is known as a "zygospore" (zygo = yoke, spore = seed) and is a true *sexual* spore. They are very thick walled and slow to germinate. Cultures (strains) which were passed around from one laboratory to another produced these spores but they sometimes suddenly lost this ability. The conditions under which the spores were produced were not understood until Blakeslee,[5] working with zygospore-producing cultures in the botanical laboratory at Harvard University, discovered the secret. Cultures which produced zygospores were really not a single fungus, but composed of two breeds or strains, in which, when they grew together, the hyphæ fused. If grown separately, there would be no zygospores and in the cultures where the function was lost, one or the other of the strains had acquired

the ascendency and checked growth in the other. Blakeslee inoculated a Petri dish with the strains to be tested and where the two strains grew together (Fig. 39, along the line *AB*) an irregular row of zygospores was produced. He obtained cultures from various laboratories in this country and Europe and matched them up to determine which were "plus" and which "minus" to his original pair isolated from the fungus obtained at Cambridge, Massachusetts. It was found that of the cultures used, 14 were minus, 6 were plus and 16 were neither plus nor minus, but were what Blakeslee termed *homothallic*. (It should be stated that *Rhizopus nigricans* has a number of relatives where zygospore production is as common as that of sporanges.) Although Blakeslee refrained from saying definitely that these strains were male and female, referring to them simply as *plus* and *minus*, he considered them in his latest papers[8] as true sexual strains which are strictly dimorphic and do not revert, that is, the sexuality is a permanent racial factor which it is impossible to change, and not a physiological condition induced by temporary nutrition.[5,7]

Blakeslee earlier considered that these fungi showed a primitive form of differentiation[5] into male and female, the two strains being morphologically very similar,

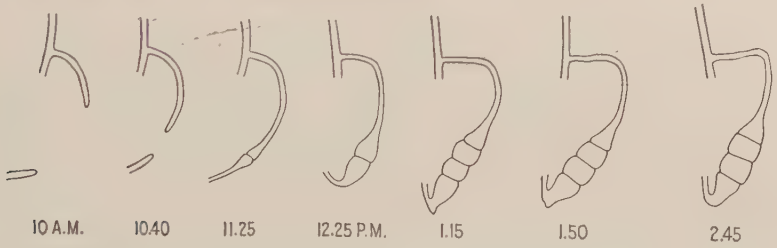

FIG. 40.—Growth of zygospore of *R. nigricans* between 10 a.m. and 2:45 p.m. (*After Blakeslee.*)

although one usually grew more rapidly than the other, a condition which might have been induced by a difference in nutrition for many generations. Each of the strains might be partially lacking in certain substances which the other strain could supply.

Zygospore Formation.—The details of the formation of the zygospores are as follows: The hyphæ touch and, as the result of the contact, a stimulus goes back along the filament and the food material flows into these ends. There is no hole through from one hyphæ to the other, so that the beginning of the stimulus must be transmitted through the cell walls. Cross-walls are now laid down cutting off the two ends (Figs. 39, 40), so that young zygospores at this stage have at least three walls across them. The two fusing ends are the *gametangia* (gamet = one of a married couple, tangia = touch). The middle wall breaks down and the growing spore is held between the two *suspensors*. One suspensor is usually much larger than the other and one side furnishes much more material than the other. A thick brown coat with irregular markings is formed around the zygospore, the *exospore* (exo = outside).

The Fusions inside the Zygospore.—The very dark thick wall of the zygospore makes it impossible to see the changes which occur in fusion and the thick wall makes it very difficult to preserve, in a normal condition, the contents in any of the ordinary fixing solutions. The consequence of these difficulties has been that the accounts do not agree either in details or essentials. All observers affirm that some of the nuclei which enter the zygospore grow and divide; others, degenerate and die. No two observers agree as to whether the nuclei from the two gametangia fuse in pairs;[18,19] whether some fuse and the others die; or whether some die and a pair fuse. The account given by Miss McCormick[16] seems very reasonable for *Rhizopus nigricans*, since it is similar to the facts that have been worked out on a number of related

Phycomycetes. As the material streams into the two ends of the suspensors, it forms a dense accumulation on either side which seems to be different chemically since it stains differently. Walls are formed cutting off the gametangia, but a central pore is left allowing communication with the two suspensors. The wall which separates the two gametangia may swell up and then disintegrate. The nuclei increase in size after the central wall disappears and all the nuclei except two, which lie at the center of the spore in a mass of denser material, disintegrate. These two nuclei fuse, apparently, although fusion was not observed. The oil in the young zygospores is in small globules, but as the spore ages, it collects into a very large central droplet which pushes the protoplasm to one side so that the spore content is largely this droplet of oil.

Germination of the Zygospores.—The zygospores of this mold are very difficult to germinate and Blakeslee[5] has unfortunately never been able to use them for studies

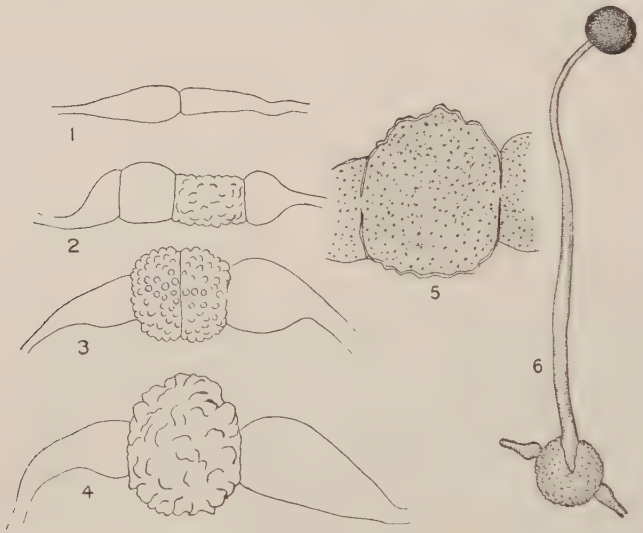

FIG. 41.—1 to 4, formation of zygospore in *R. nigricans;* 5, nuclei in spore and its connection with suspensors; 6, germination of zygospore of *Mucor mucedo* into sporangium. (1, to 4, *and* 6 *after Brefeld,* 5 *after Leger.*)

on the generation which comes from the zygospore. He succeeded twice in germination; once in 1906 when the germ tubes produced small sporangia but the spores which arose grew into weak mycelia which died, and again in 1913 when the zygospore germinated at once into a germ tube without developing sporangia. However, he did succeed with a number of other Phycomycetes. The germination of *Mucor mucedo,* a similar form occurring on dung, is shown in Fig. 41. The interesting feature of the germination is the fact that the germ tube grows only a short distance and then forms a typical sporange. The advantage to the mold in dividing up the carefully hoarded protoplasm is evident; some of the numerous asexual spores may find a suitable place for growth, while with only one the chances would be much reduced.[6]

Blakeslee investigated the spores formed in sporangia coming from the zygospores to determine the relative number of plus and minus strains which would come from one of these zygospores. Theoretically, they should have been approximately equal. The zygospore of the *Mucor mucedo,* a heterothallic form, gives rise to a sporangium in which the segregation of the sexes is only partial; not only plus and minus but also

homothallic mycelium is formed from them. These homothallic forms are not stable sexually and may revert and segregate in later sporangia into *plus* and *minus* strains.

There may be two types of zygospore germination; one in which all the spores are of one sex, the other where the spores produce mixed sexes, some spores being male and the others female.

Sexual Morphological Differentiation in the Phycomycetes.—One of these strains always grows more abundantly than the other, the mycelium being denser and the sporangia apparently larger and more abundant. Miss Dunn[9] finds that the two races she tested gave equal weight of mycelium when grown on a medium in which the source of carbon was glucose; the male (minus) race produces a greater abundance of sporangia in good solutions than does the female (plus), and the male race does better than the female so far as weight is concerned if the source of carbon is glycerine, a less assimilable carbon compound.

In a related mold, *Phycomyces nitens*,[20] the morphological sexual differences are constant for the two strains.

THE PHYSIOLOGY OF THIS MOLD

The Longevity of the Spores of Fungi.—No data seem to be available as to the length of time that the spores of *Rhizopus nigricans* or other fungi will survive under ordinary conditions but they live much longer than is generally believed. Blakeslee[5] found that the spores of *Mucor mucedo* would still germinate after 3 days' treatment with 0.4 per cent HCl, 5 weeks' immersion in absolute alcohol, or 6 weeks' drying over sulphuric acid.

Even these rather drastic treatments were not as severe as those to which the spores of various Mucors and Ascomycetes were subjected by Becquerel.[3] Spores of *Mucor mucedo*, *Mucor racemosus*, *Rhizopus niger*, *Aspergillus niger*, and *Aspergillus glaucus* were dried very slowly in small sterilized glass tubes in the presence of anhydrous baryta for two weeks at a temperature of 35°C. The air was then all taken out of the tubes with a mercury vacuum pump to a pressure of less than 0.001 millimeter and the tubes sealed airtight in the flame. After remaining for 12 months in this condition, the tubes were subjected for 3 weeks to the temperature of liquid air (−180°C.) and then, without warming, to that of liquid hydrogen (−253°C.) for 77 hours more. They were then kept 13 months more, or 25 months altogether from the beginning of the experiment, opened carefully, and the spores sown into liquid media. It would seem that after such a length of time and such treatment that none of them would have survived but the spores of the Mucors germinated in 16 hours and those of the Ascomycetes in 3 days with development entirely normal. It will be noticed in this experiment, however, that the air being reduced to a minimum, respiration would necessarily be very small, and that the slow drying would reduce the water in the spore protoplasm to the very smallest amount upon which life could be sustained. A small percentage of water enables spores to resist extremely low temperatures successfully.

The Chemotropism of Hyphæ.—Spores of Rhizopus germinate in pure water but the germ tubes which are put out will have, however, only the stored food and protoplasm with which to build until they can grow into a more favorable situation. The hyphæ must turn from a region of little food into a region of more food. Such a growth movement is called *chemotropism* (chemo = chemical, tropism = bending) and may be either *positive*, if the growth is toward the chemical, or *negative*, if away from it.

These chemotropic movements of the hyphæ of *R. nigricans* have been studied, since the spores and hyphæ are large and the fungus grows easily in culture media. The best method that has been devised for determining the attraction or repulsion of

a chemical on the fungus is by minute holes in a mica plate laid over a layer of agar containing the substance to be tested.[17,10,12] If a chemical is held in solution in the agar, it will diffuse upwards around the holes. If the fungus spores are spread in the layer above a plate with holes, the germ tubes will be attracted or repelled by the chemical which comes up through the hole and will grow either toward or away from the source of the diffusion (Fig. 42). It would seem to be very easy to determine such a bending, but, as a matter of fact, it is really very difficult to see positive chemotropism; it does not seem to be very marked for pure chemicals, although more so for plant juices. Negative movements are more pronounced and after the fungus has grown on a solution, the movements are markedly negative. This strong negative chemotropism to the liquid in which the fungus has already grown suggests that the hyphæ avoid a region in which there has already been fungus growth and that the majority of the marked reactions of a fungus are negative ones, avoiding positions

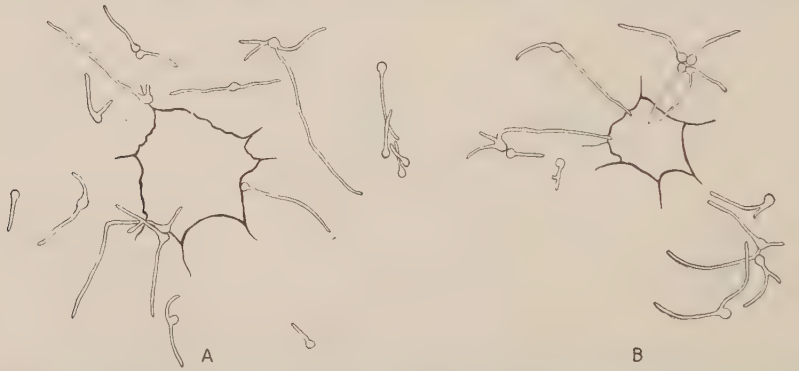

FIG. 42.—Chemotropism of germ tubes of *Rhizopus nigricans: A*, negative, growing away from a hole in a mica plate placed over staled turnip juice, the spores being in unstaled juice above the plate; *B*, same, but juice has been boiled and tubes are growing directly toward hole. (*After Graves.*)

already poisoned by the excretion produced by its own metabolism. The parasitic forms which live on or in plants spread by avoiding the regions where the growth has poisoned the food materials. These injurious products can be destroyed by heat and the fungus then grows into the remaining food substances still in the solution.

Fungi will penetrate apparently solid limestone or bone into the food materials which are slowly diffusing through them. Lind[14] made very thin sections of limestone, bone, or tooth and was able to show that if food (such as a 5 per cent sugar solution) were placed on one side of them and the fungus spores sown in water on the other, the hyphæ would penetrate them. The food diffuses through the very thin plate of stone or bone; the fungus fastens itself by holdfasts developed from the growing hyphæ; secretes acids which gradually dissolve the calcium compounds; and then pushes its way through. This does not occur if food and spores are placed on the same side of the plate. The acids produced (believed to be carbonic and oxalic) help, but the fungus seems also to penetrate mechanically. These thin sections probably have small pores in them and the fungus is attracted to the holes and enlarges them in the manner described.

Effect of Gravity and Light on Rhizopus Nigricans.—One of the older investigators of this fungus, van Tieghem, claimed that it showed neither *geotropism* (geo = earth, tropism = bending) nor *heliotropism* (helio = light, tropism = bending), but a later student[21] of the fungus found it to grow toward the light in 24 hours if clear glass were

used, or in 4 days toward yellow light. Red rays caused the fungus to grow obliquely down into the media, while green rays retarded the growth and caused smaller sporangia. It is difficult to see any advantage to a fungus of growth into white light. With a green plant, the white light would be necessary for the formation of starch, but, for fungi, light is generally considered to be detrimental.

GROWTH IN THE HYPHÆ OF RHIZOPUS

The hyphæ of a number of varieties of molds, among them Rhizopus, have been examined to determine the point at which growth (elongation) occurs. The distance between the branches does not increase, as found by measurements,[25] but the terminal portion lengthen rapidly, so that molds have only *apical* growth.

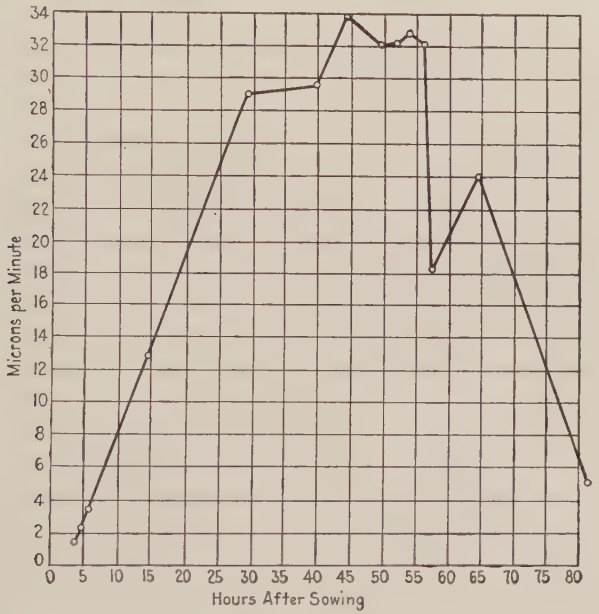

FIG. 43.—Curve showing rate of growth of a fungus (Botrytis) hypha tip at successive time intervals. (*After Smith.*)

The elongation of the hyphæ at the tip is rather slow at first, increases to a maximum which remains constant for a time, and then slows down (Fig. 43). Smith's explanation is as follows: food is being absorbed all along the hypha and is passed forward to the growing end where it is increasing in length. With elongation of the hypha, the absorbing surface increases, the amount of food available for the growing tip is greater, and the elongation is rapid. When the mycelium becomes very long, however, the difficulty of transporting the food from the remote regions in which it is absorbed enters as a factor and the elongation at the tip slackens in rate. The older portions of the mycelium are very strongly chitinized and it is doubtful if they can absorb as well as they do when younger. It is very probable that the larger portion of the absorption is also carried on by the advanced portions of the mycelium where the walls are thin and permeable; the real cause of the slackening of growth is cutinization and loss of permeability.

OTHER PHYCOMYCETES, RELATED TO RHIZOPUS NIGRICANS

Many closely related molds are often found on dung or decaying vegetation. Many of the species do not develop the runner-like growth characteristic of Rhizopus and are called *Mucor*. Another very interesting species is sometimes found on the underside of mushrooms where the gills or pores are overgrown by a golden yellow, cobweb-like mycelium. This species, *Sporodinia grandis*, readily forms zygospores of very large size.

The ordinary black mold of bread has other relatives which cause plant and animal diseases of considerable importance, as mild parasites at times on sweet potatoes, strawberries, and potatoes, and on goldfish and trout in aquaria. On sweet potatoes, one of the Rhizopus group produces a storage rot of considerable importance, while the worst enemy of the strawberry shipper is induced by another species causing "strawberry leak."

Sweet-potato Rot (Rhizopus tritici).—This disease will actively rot sweet potatoes in storage, forming diastase in the mycelium of the fungus and even after several months storage at 9 to 35°C. it still converts starch into sugar, the young mycelium containing the most active enzyme.[13]

The same variety of Rhizopus[27] will rot half of an inoculated sweet potato in 3 days and from 6.3 to 7.8 times as much carbon dioxide is given off from the rotting half as from an uninoculated half. An analysis, at the end of the experiment, of the rotted half of the tuber indicates that even more carbohydrates had been destroyed by the fungus than was indicated by the amount of carbon dioxide given off, the other carbohydrates having been used for other purposes, such as the building up of the fungus proteins or the production of alcohols and acids. The amount of carbon dioxide evolved by a fungus is never a true measure of the amount of carbohydrate lost (see p. 47), since some of the carbon must always be transformed into the proteins and reserve food stuffs of the fungus itself.

Strawberry Leak and Rot.—Infection occurs through small wounds in the berries, the fungus being unable to pierce the unbroken cell walls. (1) Berries picked early in the morning, even if they are covered with moisture, are less likely to rot and ship better than those which have been picked later in the day after the berries have been warmed by the sun. (2) Of the shipments of berries which showed rot, over two-thirds of the loss was due to infection with Rhizopus, according to Rose[23] who examined the data gathered by the U. S. Department of Agriculture at terminal markets.

Some relatives of *R. nigricans*, but not so nearly related, are the cause of disease in plants and animals.

The Damping-off of Seedlings.—The damping-off of very young seedlings in seed beds or in the greenhouse often results from the attack of one of the Phycomycetes, *Pythium de Baryanum*, a fungus which lives just under the surface of the soil whence it makes its way into the tissue of the young tender plants. The water supply of such plants is shut off, the fibrovascular bundles are filled with fungus threads, and the plant topples over and wilts.

The Late Blight and Late-blight Rot of Potatoes.—The most serious fungus enemy of the potato is the late-blight fungus, *Phytophthora infestans*, another Phycomycete, which, in the Northern States and in Europe, invades the tissues of the leaf and rapidly rots them in humid cool periods of weather. The plant is finally left standing with practically all of its foliage gone, or the stems also may become infected and succumb. Spores of the fungus are washed down into the soil, penetrate the skin of the potato tuber, and cause the crop to rot in the field. The great Irish famine of 1845 was due to the loss of the Irish potato crop by this fungus.

The White Mold on Goldfish.—The white growth appearing on injured goldfish is due to a water mold which locates itself on the flesh of the fish where the scales have

been removed and grows into the flesh of the body, usually killing the animal after a time. A similar disease affects, at times, young trout and other fish in hatcheries.

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CHAPTER XI

THE MORPHOLOGY AND REPRODUCTION OF THE BLUE-GREEN, GREEN, BLACK, AND YELLOW MOLDS

ASPERGILLUS AND PENICILLIUM

The Aspergillus and Penicillium groups of fungi are characterized by spores borne in chains on a special branch of the mycelium. Such a spore is known as a *conidium* (dust) and the stalks are known as *conidiophores* (conidio = dust, phore = bearer).

FIG. 44.—Reproduction in *Aspergillus repens* (Eurotium repens). *A*, *B*, asexual reproduction, conidia borne on short sterigmata on long conidiophores; *B*, conidiophore and sterigmata in section. At the right of *A*, twisted beginnings of perithecium. *C*, *D*, and *E*, further stages in development of perithecium, in section in *F*; *G*, ascus with eight ascospores, only seven shown; *H*, one ascospore of *A. glaucus*. *A* and *B* × 375; others × 1,500. (*After de Bary.*)

The spore formation is always basal. The youngest spore is cut from the conidiophore as a small short cell which continues to grow for some time but which is pushed outward by another younger cell underneath it, to be succeeded as the youngest spore by still another one; the oldest one is pushed gradually away from the conidiophore. In this manner, a long chain of individual conidia may arise (Figs. 44 and 45).

The basal conidia in Penicillum may have a cylindrical or barrel shape (Fig. 45), but the older ones round up and form new walls inside the old one. The connective between them is the old wall, which may assume a variety of shapes, or apparently not be present at all.[21] The cell which gives rise to the conidia is known as the *conidiophorous cell* or *sterigma.*

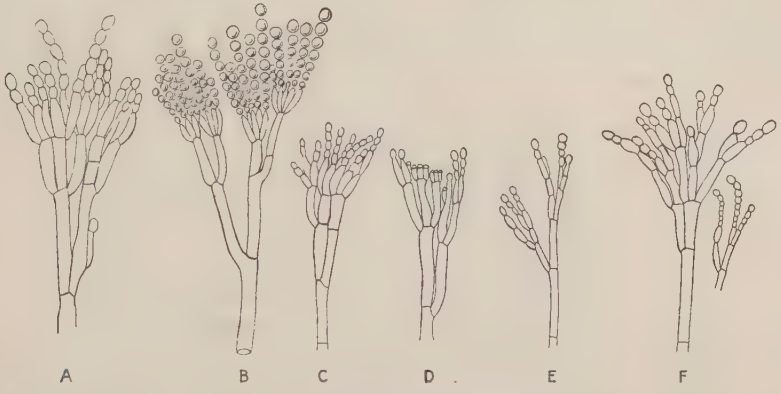

FIG. 45.—Various species of Penicillium. *A, P. commune; B, P. glaucum; C, P. italicum; D, P. expansum; E, P. roqueforti; F, P. camemberti.* (*A, C, D, E, and F are after Thom; B, is from Brefeld.*) The *P. glaucum* is shown because the name frequently appears in the literature, but the drawing is too diagrammatic to be conclusive as to species.

In Aspergillus the radial growth of the chains of conidia from the *sterigma* form a roughly spherical head; in Penicillium, the growth from the brush-like ends of the conidiophores produces a broom-like structure. The spores at the ends of the long chains are removed by the wind or are brushed off, as the cell connections between the

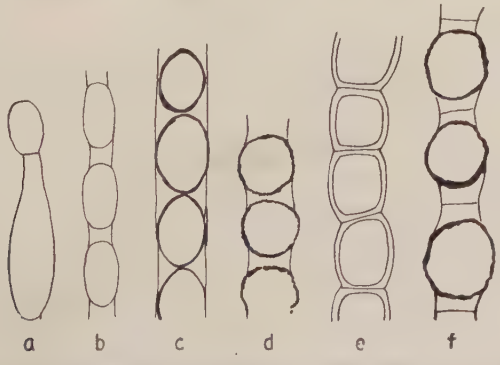

FIG. 46.—Conidium and connective formation in Penicillium: *a*, conidium-bearing cell showing tube and conidium in elliptical stage; *b, c, d,* chains of conidia showing different appearances of the connectives but no cross-walls; *e, f,* chains of conidia with original cross-walls. (*After Thom.*)

end conidia are probably very weak. The long stalk which bears the small branches in Aspergillus is the conidiophore, the short branches themselves are the sterigmata (Fig. 44*A, B*).

Coremia.—The aggregations of hyphæ of *P. expansum* on apples are called *coremia* (singular, *coremium* = broom). While the formation of coremia is especially charac-

teristic of the apple rot Penicillium, they may be found occasionally, also, on cultures of the common green molds from the air. The coremia are little structures made up of fungus hyphæ, white on the sides but green with spores at the apex, with a height of about a millimeter above the surface of the fungus growth. Wächter[24] believes that the fungus attempts to get above its watery substratum by the formation of stalks which lift it into a less humid region where spore formation may be carried on under more normal conditions. His explanation of coremia formation in *P. expansum* on the apple is that the surface of the apple, being thick and waxy, is not adapted to the formation of a broad cover of spore-bearing hyphæ, so the fungus developes coremia in

Fig. 47.—Cytoplasmic and nuclear structure of Aspergillus. *A* to *C*, developing conidiophore showing development of sterigmata and conidia; *D*, sections of mature conidia with various sizes of nuclei; *E*, portion of vegetative mycelium with numerous nuclei, *A to C* × 1,500; *D*, *E* × 3,700. *A to C, after Fraser and Chambers; D and E, after Dale.*)

order to increase the available spore-bearing area. The fungus does not grow well on the surface of the apple itself and only breaks through in spots at the lenticels or breathing pores of the fruit.

The mycelium is relatively small in diameter, with many cross-walls. Van Wisselingh[23] and Wettstein[25] were able to get a pronounced positive reaction for chitin in the cell walls of both the green *Aspergillus* and *Penicillium*, not only in the mycelium but in the conidiophores, the sterigmata, and the conidia themselves, although they were apparently unable to get a test for cellulose.

The green or blue-green color is given by the very faint color of the plasma.[4]

The fat droplets which appear in the hyphæ of *A. niger*, as well in those of many other molds, are to be regarded as stored up food similar to that of the animal body. Dubaquie[12] observed that they are most numerous on the second day after spore

germination, after which they diminished with the increasing dry weight of mycelium. As the mold begins to form spores, fat is stored as food for the young plants which they produce, being a material of high calory value and not readily decomposed under unfavorable conditions.

Various substances have been found in the body of molds such as *Aspergillus niger*, among them being pentosans,[11] the physiological significance of which is not clear.

The Cell Structure.—The conidia and the mycelium contain many nuclei in every cell so that Aspergillus is a *cœnocyte* (Fig. 47). The conidiophores arise as dense

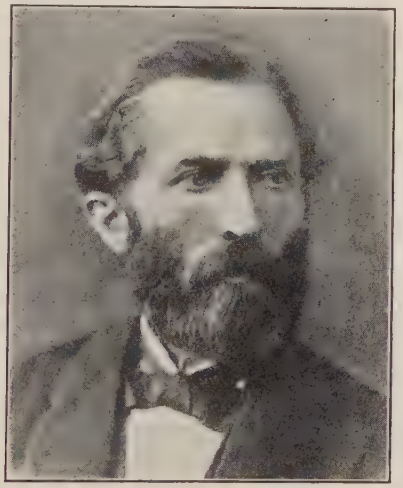

FIG. 48.—Heinrich Anton de Bary (1831–1888). An early investigator into the life histories of fungi and bacteria.

swollen hyphæ above the mass of vegetative strands. The rounded and swollen end contains many nuclei and, as the sterigmata are formed, some pass out into them and thence into the young conidia.[9,16] The nuclei vary in size in the mycelium, as is shown in *D*, so that they can hardly be regarded as being all potentially equal.

Sexual Reproduction in These Molds.—In addition to the methods of strictly asexual reproduction just described, fusions may be found at times inside masses of hyphæ, wound up in a tangle to form a small ball-like structure. These masses of hyphæ are called *sclerotia* (singular, *sclerotium* = hard) or *perithecia* (singular, *perithecium*; peri = around, thecium = covering). Inside the perithecia are sacs, *asci* (singular, *ascus*), containing another type of spores, the *ascospore*, usually eight being found in each ascus, although the number may vary from five to eight.

The Sexual Spores in Aspergillus.—We owe the discovery of these capsules to de Bary, in 1859 (Fig. 48), who, by using cultures of the common green species of Aspergillus, was able to demonstrate that the fungus which had been known to mycologists as *Eurotium repens* or *E. herbariorum* was only one stage in the development of a common green mold known as *A. glaucus*. This discovery of asci and ascospores removed Aspergillus from the large nondescript group of the *Fungi Imperfecti* into the Ascomycetes.

The perithecia originate as spirally coiled hyphæ which develop an outer protective wall (Fig. 44*C-F*) with an inner tangled mass of hyphæ with swollen ends, the latter being cut off to form asci, *G*. The ascospores are formed, internally, inside the ascus, by a cutting up of the material which it contains by a process known as *free-*

cell formation. The perithecia are yellowish brown at first and gradually become a darker brown as they age.

Some strains of *A. flavus* and *A. niger* produce *sclerotia*, sterile bodies composed of mycelium only.

Sexual Fusions in the Cells in Aspergillus.—The question of male and female gametes is very doubtful, but fusions[9] occur in pairs in the much swollen cells which give rise to the asci (Fig. 49*A-C*). This fusion inside the cells means that the nuclei are all of one sex and that the whole process is a reduced form of sexuality. The

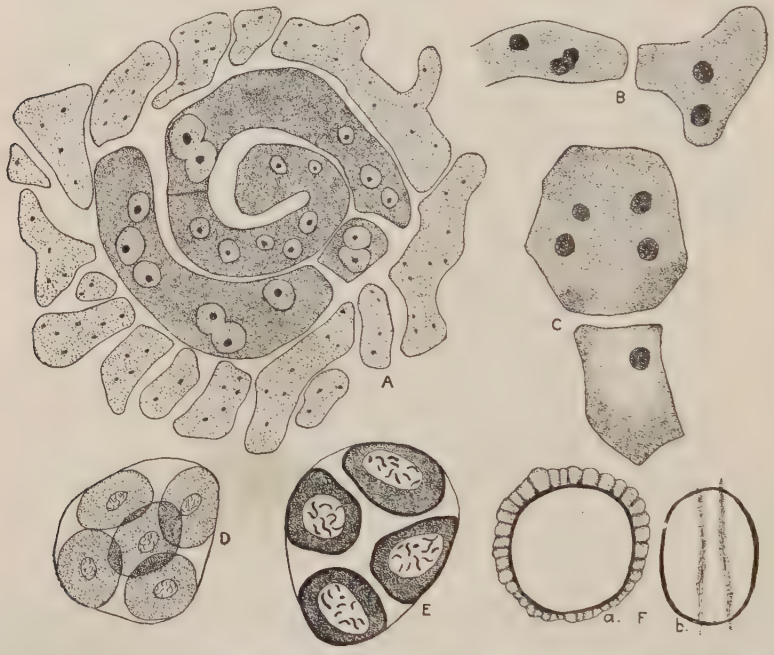

FIG. 49.—Cytology of ascospores in *A. repens: A*, young ascogonium with some transverse walls, nuclei fusing in pairs; after fusion the nuclei divide to form nuclei of ascospores; *B*, nuclei divided once so that each cell has two nuclei; *C*, each cell has four nuclei; *D* and *E*, young ascospores; *F*, mature ascospores in side and end view. *A* × 3,500; *B* to *F* ×5,200. (*After Dale.*)

fusion nuclei pass out into the hyphæ which will form the asci and there undergo a series of divisions, *C*, so that each ascus has eight nuclei, as a rule, although two and four occur. Each of these eight nuclei takes a little of the cytoplasm of the ascus and rounds up into a thin-walled spore which, gradually, changes into a thick-walled one, *D-E*. The ascospores are uninucleated. In some species, such as *A. herbariorum*, *F*, the ascospore shows the markings characteristic of it, but in *A. repens* the walls are comparatively thin and smooth.

Reproduction in Penicillium by Sexual Spores.—While Penicillium ordinarily reproduces only by means of conidia, at times the fungus also forms perithecia and ascospores, but very rarely, and only discovered by Brefeld[5] (Fig. 50), a long time after de Bary had observed them in *Aspergillus glaucus*. Although Leveille[19] had seen sclerotia in a Penicillium growing on some very old tamarinds, he had paid little attention to them. The discovery is still doubted by many mycologists and the question which the modern systematist asks is, What Penicillium did Brefeld have?

His discovery, if correct, was a distinct advance in our knowledge of the fungus and his production of perithecia in cultures very ingenious. He sowed Penicillium spores on a slice of fresh wheat bread, moistened the bread with a little distilled water, and placed it under a bell jar in the laboratory. The bread was wet with water again

FIG. 50.—Oscar Brefeld (1839–1925). Student of the fungi and bacteria.

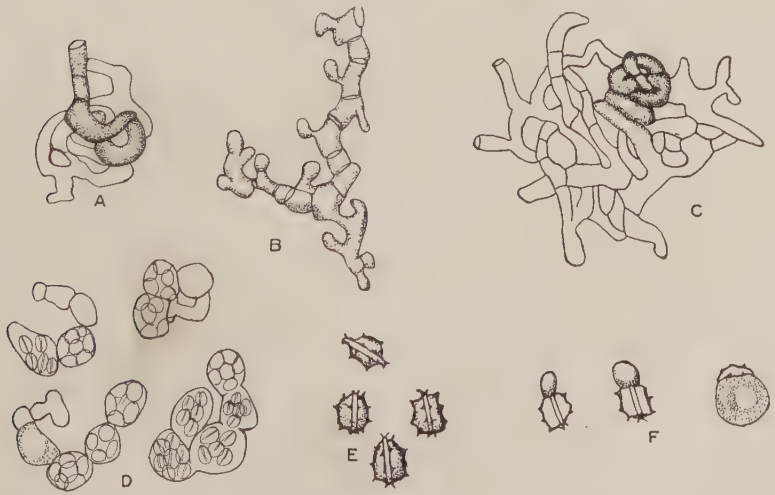

FIG. 51.—Formation of asci and ascospores in Penicillium: *A* to *C*, stages in the formation of the young perithecium; *D*, asci with ascospores; *E*, ascospores; *F*, germination of ascospores. (*After Brefeld.*)

on the third day and on each succeeding day until the sixth or seventh day when it was taken out from under the bell jar, no conidia having as yet developed, and placed between two glass plates so as to prevent all access of oxygen. The sclerotia began to appear within 24 hours after this treatment, the youngest stages which could be observed (Fig. 51) showed two twisted hyphæ placed on a thick branch. These

spiral hyphæ seem to be of different material from that of the mycelium in general, and their two ends seem to fuse to form some type of sexual structure out of which grow the tube-like projections upon which are borne the asci. The thick-walled hyphæ surround the spore cases as a dense mass of sterile cells (Fig. 51). The interior of the sclerotium becomes filled with many asci as its walls become dark brown. The sclerotia (or perithecia) can almost be seen with the naked eye since they are from 0.165 to 0.870 millimeter in diameter (Fig. 52). They are so hard that they cannot be crushed readily under the cover glass. The production of sclerotia is, according to Brefeld's method, induced by an absence of oxygen. While other investigators have tried to repeat his experiments, using his methods, without success, Brefeld developed

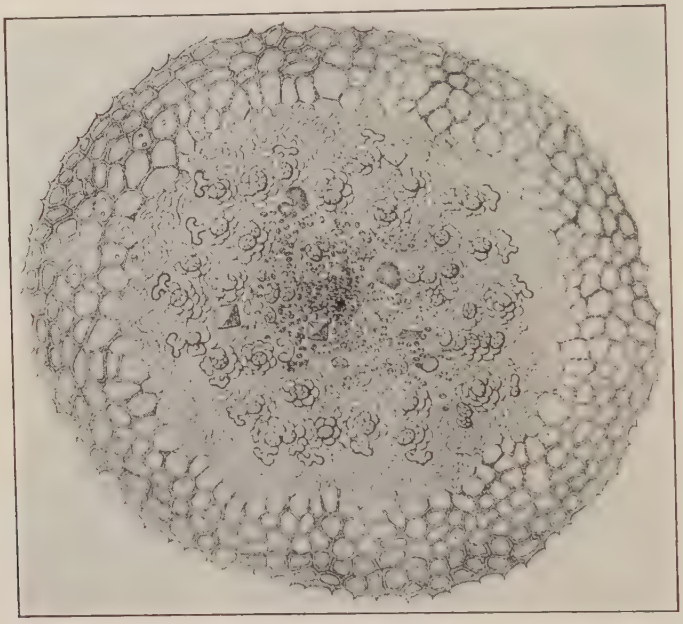

Fig. 52.—Asci and ascospores of *Penicillium glaucum* from bread kept in partially anærobic conditions by enclosing the piece of bread between glass plates after fungus had started to grow. (*From Brefeld.*)

such an abundance of material that he convinced the scientific world; the question as to species, however, is still unanswered. The absence of oxygen suggests that the perithecia are formed in the interior of foods on which the fungus is growing, the mold-producing conidia on the outside, and ascospores in the interior. From what has been learned, too, regarding strains of fungi, and the part which several strains play in the sexual fusions, the very plausible theory presents itself that Brefeld may have been using two strains of the mold, a *plus* and a *minus*, to use the terms applied by Blakeslee to the Mucors and other Zygomycetaceæ.

It is to be further noted that the process is a sexual one, the result of the fusion of two hyphal branches, and that while the spores do not arise from the sexual product directly, they do come from branches which have their origin in the cells which have fused, so the spores produced are to be regarded as *sexual*, in contrast to purely vegetative conidia.

Brefeld claimed to produce the same Penicillium when he germinated the ascospores after 18 to 24 hours in prune juice which had been sterilized, the mycelium

coming from germinating ascospores (Fig. 51), resembling in every way that produced by the germination of the conidia, and in 2 days themselves producing conidia of the Penicillium type. Ascospores retain their vitality for several years but not any longer than the ordinary conidia, which remain capable of germination after 1¼ to 1¾ years in the air of the laboratory.

THE COMMON ASPERGILLUS AND PENICILLIUM MOLDS

Naming Species of Molds.—The taxonomist depends first on the comparative form and size of various plant organs. In all very simple forms, however, the plant has very few parts which can be described, and two or more apparently different plants seem to be exactly the same under the microscope. Recourse then, must be had to the growth, and products on various culture media.

The *morphology* of the Penicilliums is so similar that it has been found possible to make but a limited use of it,[27] and the cultural characters vary and are not dependable. A number of the species cannot be distinguished by any marked morphological characters but there is the possibility that they may be differentiated according to their *physiological* reactions.

Some strains may have a physiological property very strongly developed, others less so, and in some, it may be almost entirely lacking. The question then arises, How much weight can be given to a single physiological character? Does its presence or absence make a distinct species? The answer differs with characters and with their apparent importance. Usually, two or more characters must be different to make a distinct species. All the green molds were known as *P. glaucum* to Link in 1824 but the presence of coremia set the Penicillium which rots apples, *P. expansum*, off from the other green species. Its general occurrence on apples is another distinguishing character; but other green varieties also form coremia, so that other characters must be studied in order to separate two coremia-forming varieties. When morphological species are subdivided into unstable and innumerable physiological ones, we are all at sea. The old landmarks of morphology are certainly deceptive enough and not constant in these lower forms, but they are gleaming lighthouses as compared to the evanescent physiological characters, many of which may be changed or lost.

The same difficulties which Woltje[27] and Thom[20] met in Penicillium are to be found in Aspergillus and it is doubtful if we have arrived, as yet, at any completely satisfactory classification. The following species seem, however, to be fairly easily recognized with characters that are constant.[20,22]

Aspergillus niger is the common black species found everywhere in nature and occasionally slightly pathogenic on fleshy plants, such as summer squash. It grows readily in culture media and has been used as the object of experimentation by many investigators of fungus nutrition, respiration, and the influence of various physical and chemical stimulants and poisons.

Aspergillus flavus is another very common mold of yellow color with a shade of green in it. It grows best at warm temperatures (37°C.) but is common in the laboratory on cultures and in the home on preserves, jams, etc.

Aspergillus oryzæ, a member of the *A. flavus* group, is a common Japanese mold which has great economic importance as a producer of diastase. It is a yellowish-green mold easily cultivated in the laboratory.

Aspergillus fumigatus is a widely spread species, of interest because it becomes pathogenic at times on men and animals. It grows and fruits in the bronchiæ of birds, such as the pigeon.

Aspergillus glaucus is the commonest green mold, found everywhere through the world. The young mycelium is a light-green color but as it ages, turns to a dark gray-green color or even a brown. The young conidiophores are to be found on the margin of the colony where the color is light green.

Penicillium glaucum is the old name given to green molds of the Penicillium type, although the species probably included numerous varieties. It is everywhere present in nature and will grow on almost any substance. The conidia are always spherical in form.

Penicillium roqueforti is the mold which ripens Roquefort cheese and which may be found scattered in a fruiting condition in the holes of this type of cheese. It is a green mold, once supposed to be the same as *P. glaucum*, but differs from it in the larger conidia and in the enzymes by which it digests the casein of cheese.[10] The mold is grown on bread crumbs and mixed with the curd obtained from sheep's milk. The mold grows inside the cheese, in the holes, the introduction of air being further developed by the piercing of the cheese with small needles. The cheese is ripened in France in caves where the temperature is about 15°C. These caves, being damp, are ideal curing rooms for the growth of the mold and the secretion of its enzymes.

The very piquant flavor which is so characteristic of Roquefort cheese is due to a series of volatile acids produced in the cheese by the hydrolytic action of the mold upon the fats. Well-ripened cheese contains[8] about 45 per cent of the volatile acids, the principal ones of which are caproic acid, 13 per cent; butyric acid, 19 per cent; and acetic acid, 5 per cent. The pepper taste of the cheese is due to the caproic, caprylic, and capric acids, all of which occur in the ripened cheese.

Penicillium camemberti is the mold found on the exterior of Camembert cheeses, usually seen as a dirty white-colored mat surrounding the digested contents. This cheese is very soft, containing 50 per cent of moisture. The curd is ripened first by the lactic-acid organisms but after 5 or 6 days *P. camemberti* begins to grow on the outside of the curd. The curd is from cows' milk. The mold renders the cheese less acid and secretes enzymes which penetrate it and gradually soften the interior.[10] When they have reached the interior, the cheese is ready for consumption and must be used within a short time or putrefying bacteria will become the predominant type of organism.

Penicillium italicum is one of the bluish-green molds causing rots of oranges and lemons in storage. It was originally isolated from oranges from Italy but is found throughout the tropical world. The mold is the blue-green form species on citrus fruits, the darker color being surrounded by a zone of white.[20]

Penicillium digitatum is another common citrus fruit parasite, the colonies being not blue-green but olive-green. It may occur on the same fruit with the *P. italicum*.

Penicillium expansum is the storage rot fungus of apples and pears. It forms large coremia composed of masses of hyphæ with the fruiting conidiophores at the tips.

OTHER COMMON RELATED FUNGI

Sclerotinia cinerea is the common fungus which appears as a gray mold on the surface of peaches, plums, and cherries, both on the trees and in the market, and which

internally produces *brown rot*. The rotting fruit can be seen hanging in clusters on the trees, the clusters, drying to form what are called *mummies*, do not drop at once from the tree. They finally drop and lie for 1 to 2 years on the ground partly covered by the grass and leaves. They germinate in the spring to form goblet-like fruiting bodies in which are borne the spores of the ascus type (Fig. 53*D-E*). These are shot up into the air and infect the young twigs or young fruit. The gray mold which appears on the outside of the fruit (Fig. 53) is composed of the conidia and conidiophores of the fungus. The conidia spread the disease through the tree and to other fruit.[13,14,18]

Aa

FIG. 53.—Brown rot of plums and peaches (*Sclerotinia cinerea*). *Aa*, as it appears on the dried fruit on the trees: mummied plums covered with conidia of brown-rot fungus. *A* to *C*, the conidia and their germination; *D*, peziza cups coming from old plum; *E*, section of peziza cup with asci and ascospores, *A* to *C* × 90; *D* × 2½, *E* × 600. (*After Woronin; Fisher and Brooks.*)

Alternaria or *Macrosporium* is very common on vegetation and in dust. It forms dark-brown colonies on agar plates. Its distinguishing character is the large many-celled spores borne on a brown thick-walled mycelium.

Cephalothecium roseum is a salmon-colored fungus in cultures, very common in dust, which also plays the rôle of a parasite when it appears on the scabby spots on apples and pears. It has a two-celled conidium (Fig. 54).

Fusarium sp. often appears on sugar-agar plates exposed to the air or on similar plates made from the soil. The fusaria are among the very common soil fungi and are at times mild parasites. They form over culture plates a cottony white growth with sickle-shaped conidia divided into three to six cells by cross-walls.

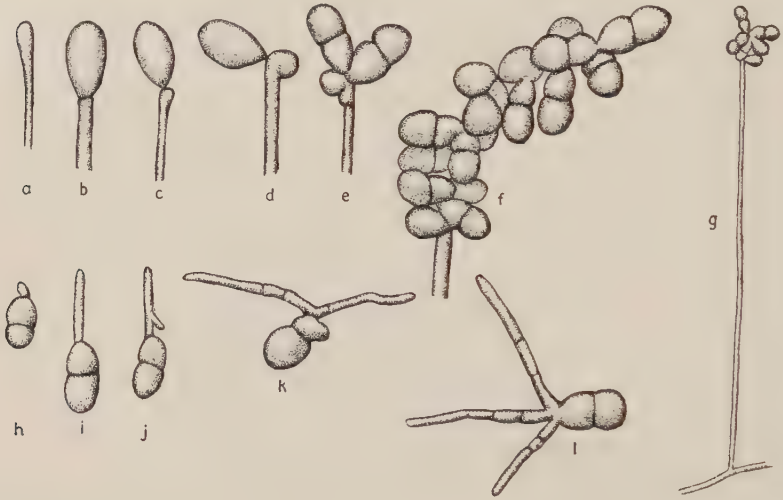

FIG. 54.—Formation and germination of spores of *Cephalothecium roseum.* The first spore is formed terminally, *a*, *b*, and *c*; but the second spore produced on same conidiophore starts at the base of first one, *c* and *d*, and pushes the older one to one side. Basal production of spores continues until a series may form a long irregular chain. *f*. Germination of spores, *h*, *i*, *j*, *k*, and *l*. (*After Van Hook.*)

THE COMMERCIAL SHIPPING AND STORAGE OF FRUITS

In the days before modern storage plants and refrigerator cars, fruit could be eaten only in the region in which it was produced, if it was of a perishable nature, and then only for the very short season during which it would remain sound when gathered from the trees. The modern storage houses and refrigerator cars, however, make it possible to eat oranges in Vermont in September just as fresh as when they were gathered from the trees in Florida in the preceding December, and Vermont apples can be eaten in Florida in May when they were picked in October of the previous year. These possibilities have been the result of a careful study of the rots which affect these fruits, and their control. The loss of water from the fruit must be checked, if it is kept for a long time, and this means a fairly humid atmosphere where rot fungi have a chance to grow. The old method of the storage of apples in cellars checks the spread of the rots (not entirely preventing them) but the refrigerating plant where the fruit is held at approximately freezing temperature prevents spore germination and mycelial growth of the rot fungi.

THE EFFECT OF TEMPERATURE ON THE GROWTH OF THE MOLDS OF FRUIT ROT

The fungus of the common storage rot of apples, *Penicillium expansum* (Fig. 55), will, in 1 week at 10°C., germinate and begin to rot the apple; at 5°C. it will not germinate before the third week; and at 0°C. only after 4 weeks.[2] At 0°C., inoculated spots were only 10 millimeters across after keeping them for 8 weeks, but the apples rotted. The packer of the fruit must depend not only on the low temperature of his storage room but also on the unbroken skin and freedom from blemish of the fruit

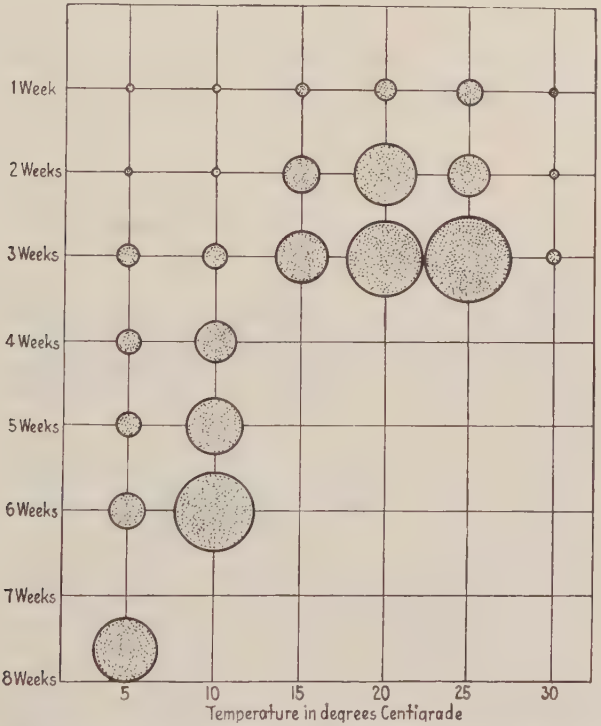

FIG. 55.—Development of rot spots caused by *P. expansum* on Winesap apples in storage at different degrees of temperature. (*After Brooks and Cooley.*)

that he puts into it, for fruit with any break in the covering will gradually rot even at 0°C.

The length of time required for the germination of the spores of some of the common rot fungi is shown in Fig. 56. *Rhizopus nigricans*, a common rot of sweet potatoes in storage, will germinate in spores at 3 to 4°C. in 200 hours; while *Penicillium italicum*, the common rot of oranges, will germinate in 125 hours at 1°C.; and *Sclerotinia cinerea*, the brown rot of stone fruits, will produce germ tubes from the spores at 1°C. but requires 250 hours. Above 10°C. the spores germinate in a few days and are capable of starting a rot spot.

Mold spores will occasionally germinate at the *high temperature* but a temperature is reached, varying with the different species, where germination stops (Fig. 57). These temperatures are too high, however, to use in the storage of fruit as loss of

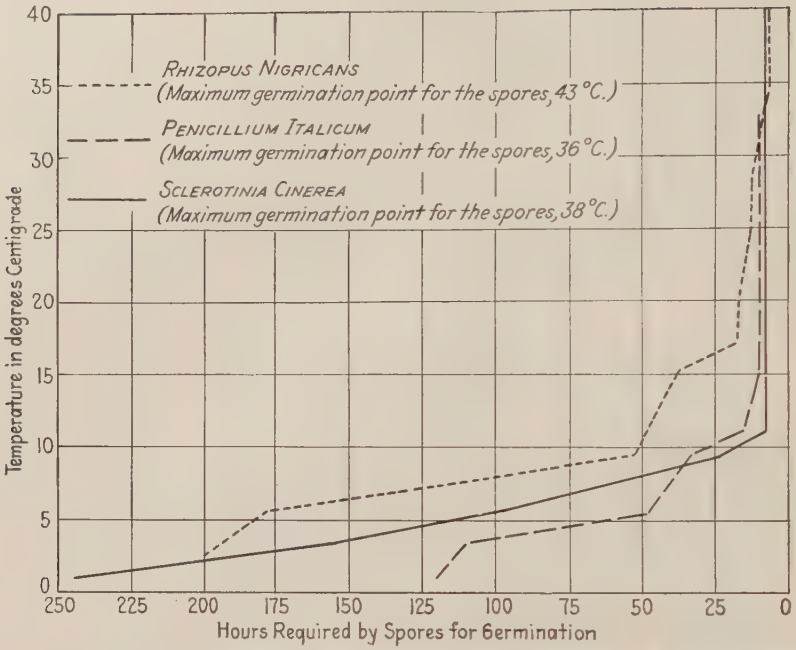

Fig. 56.—Germination of spores of various fungi which cause the rot of fruit in storage. (*After Ames.*)

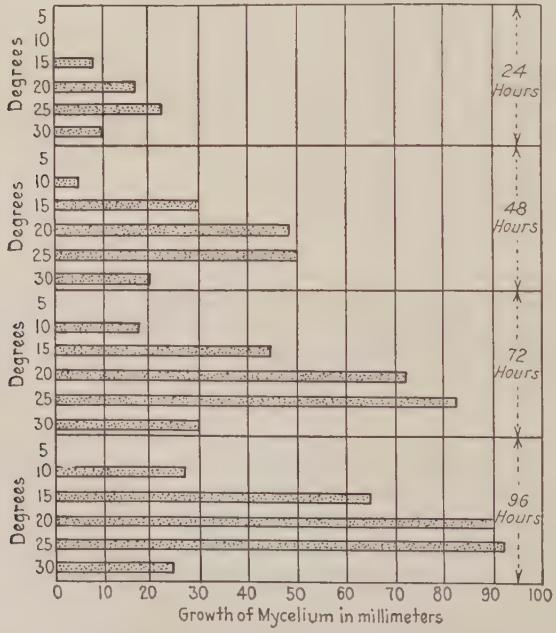

Fig. 57.—Temperature relations of 11 species of *R. nigricans;* growth of mycelium at different temperatures. (*After Weimer and Harter.*)

water and the respiration would be too high. This temperature[2] is much below the *thermal death point* of the fungus spores:

Rhizopus nigricans	*Sclerotinia cinerea*	*Penicillium italicum*	*Cephalothecium roseum*
60 to 60.5°C.	52 to 52.5°C.	58 to 58.5°C.	47 to 48°C.

The Organisms Causing the Rotting of Fruits in Storage.—The rotting of apples is due usually to the so-called storage rot, *Penicillium expansum*. This organism enters the fruit through a broken skin as it secretes no enzyme to dissolve the very thick layer of wax and cuticle which protects the flesh of the apple. After entering, however, it penetrates between the cells, dissolves out the pectin which holds them together, and a soft slimy mass of cells results. Much of the sugar of the fruit disappears and various acids are developed which change its typical flavor completely. Behrens[3] found that the rotten part of an apple was much less acid than the sound portion, the fungus apparently using up the malic acid and sugars.

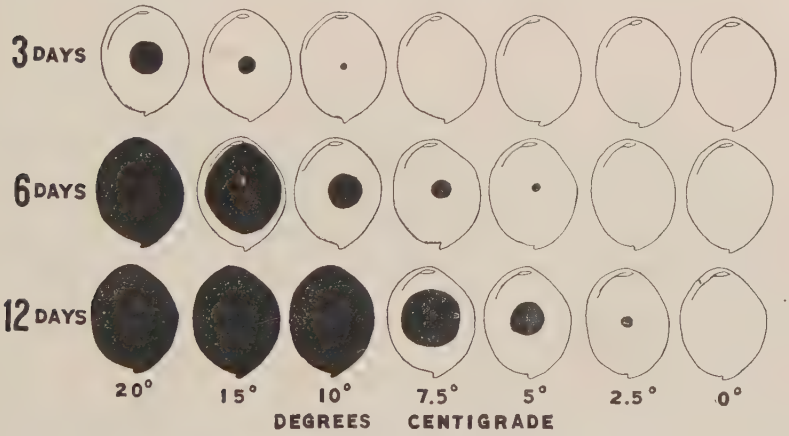

FIG. 58.—Spread of *Sclerotinia cinerea* (brown rot) in peaches in storage at various temperatures. (*After Brooks and Cooley.*)

The rotting of oranges is the result of the action of the molds *Penicillium italicum* and *P. digitatum*. The most extreme care must be taken to avoid bruising oranges or lemons in shipping, for these organisms will find food in the material which comes from the crushed cells of the orange peel and can readily penetrate this rather spongy layer; injuries near the stem end are more likely to cause rot than those at the bud end.[15] Low temperatures, care in packing, and avoiding bruising make the loss smaller, but even then the orange shipper and merchant always lose quite a percentage of their fruit. Before the fruit is packed, it is usually washed, dried, and wrapped in thin tissue paper. The tissue paper wrapping will not prevent evaporation, as would a waxed paper, but the special purpose which it serves is to prevent the spores of the fungus from spreading. The spores form on the outside of the fruit and spread the disease.

The rotting of peaches, plums, and cherries both on the trees and in shipping is due to *Sclerotinia cinerea*, the brown-rot fungus (Fig. 58). This fungus is able, apparently, to penetrate the skin of these fruits and, once inside, produces a rot like the storage rot of the apple but harder in texture, although the fungus secretes an enzyme, pectinase, which dissolves the pectin between the cell walls. Oxalic acid

seems to be a regular product of the fungus in the peach. The principal change[7] which the organism brings about is in the sugar content, the rotten parts being full of reducing sugars but showing little cane sugar. The fungus apparently inverts the cane sugar into glucose. The acid content is increased.[17] Willaman and Davison[26] found in all cases that the plums affected by the same brown rot had a higher ash content, more CaO, nitrogen, and oil or fatty substances, than the unrotted ones. In some cases the crude fiber was more abundant in the diseased plums but in other cases it was not.

FUNGI IN THE SOIL

The bacteria have received so much attention from their work in the soil that they have overshadowed the importance of the fungi as active agents in inducing changes in the organic and inorganic substances which may be added to the soil as fertilizers, or which find their way into the soil from the bodies of the plants and animals which are left there.

Abbott[1] investigated 28 species of fungi isolated from the soil to determine the changes which they might induce He found that the Fungi Imperfecti were the strong ammonifiers and, that while the Penicilliums were abundant, they were the weakest of the producers of ammonia. He found also that two Penicilliums, *P. funiculosum* and *luteum*, as well as *Aspergillus fumigatus* and *flavus*, and *Cladosporium herbarum* were able to render phosphoric acid available from the crude phosphates.

The chief function of the soil fungi, however, seems to be to destroy carbohydrates and proteins in the upper layers of the soil where air is available. They are very abundant in the first 3 to 8 inches and then rapidly diminish in numbers so that 2 feet below the surface they are scarce and at 4 feet almost none are to be found.[6]

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CHAPTER XII

THE PHYSIOLOGY OF THE GREEN, BLACK, AND YELLOW-BROWN MOLDS

The green molds grow so readily and rapidly under laboratory cultural methods and present so many interesting problems in nutrition that they have been studied intensively with regard to the following topics:

1. The enzymes through which the food is made available together with the products of the action of the enzyme.
2. The germination and poisoning of the spores.
3. The availability of different forms of carbon as the source of energy.
4. The fixation of the nitrogen of the air by molds.
5. The best source of nitrogen.
6. The effect of used culture media on fungus growth
7. The formation of rings in old mold colonies.
8. The spread of fungus spores by air currents.

THE MOLD ENZYMES

Organized and Unorganized Ferments (Enzymes).—After Pasteur[61,62] had discovered that living organisms could always be seen under the compound microscope when fermentations were occurring in sugar solutions, the yeasts or fungi producing them were spoken of as "organized ferments." On the other hand, the changes which occurred in solutions without any visible organisms were said to be due to "unorganized ferments." The unorganized ferments were the products in many instances of the organized ferments, so there soon came to be a confusion of terms which was solved by substituting for unorganized ferments, at the suggestion of Kühne,[42] the new term "enzyme" (en = in, zyme = yeast). The intention, however, was not to limit it to the yeast ferments but to include all ferments, such as diastase, which had already been discovered and named in 1833.

Enzymes are named from a combination of the substratum with the suffix "ase", thus, "sucrase" is the enzyme which would invert sucrose, although the old name of "invertase" still clings, since it more specifically indicates the type of chemical reaction. Some old names, too, have not been displaced by the new combinations, among them, *diastase* and *zymase*.

The ending "lytic" has been combined with the substance on which an enzyme acts to indicate that it destroys this particular substance,

but the construction of the word is faulty, and it has been proposed by Armstrong to substitute the ending "clastic" meaning "breaking." The enzymes which have the power of breaking up gelatin into a liquid condition would be called "proteoclastic" enzymes, therefore, instead of "proteolytic" ones. The new ending has found its way into general use in the discussion of enzymes.

DIASTASE OR AMYLASE FROM FUNGI

The action of the diastase produced by fungi is much more vigorous than that which comes from germinating barley grains (malt diastase). Enzyme of fungus origin, as compared to malt diastase, hydrolyzes starch into sugar completely and four to five times faster. It has been used in medicine as a cure for amylolytic dyspepsia, a form of indigestion where the secretion of starch-producing enzymes by the glands is too weak to take care of the starchy foods. In industry[74] it is useful in:

1. Textiles for removing size (starch).
2. Clearing fruit extracts which contain starch.
3. The manufacture of various starch derivatives.

1. Separation of the Enzyme.—Much of our knowledge of enzymes is the result of the study of the diastase from Aspergillus. The enzyme may be successfully precipated out of solution by ammonium sulphate, although alcohol is usually used.[54] The precipitate contains nitrogen, but the amount of this substance is not a measure of the activity of the enzyme contained in it, since the enzyme is not necessarily of nitrogenous composition.

Its Properties.—The addition of small amounts of sugar will not check its action; a large amount of maltose will stop it. The enzyme seems to resemble an organism in that it may be poisoned by the products of its own activity, in this case, maltose. Heating weakens the power of the enzyme to convert starch, but if not carried too far, this ability can be restored by the addition of sodium chloride and nitrogenous substances, such as white of egg, peptone, or asparagin. Heating seems to injure the nitrogenous portion of the enzyme but it may recover from it with proper nutrition. Enzymes and their action will be discussed more in detail under the yeasts.

The Commercial Production of Diastase.—The diastase, today for sale as a pharmaceutical preparation, is known by the trade name of "Taka-diastase." The process of the manufacture of Taka-diastase has been described by Takamine,[70] the Japanese chemist who invented the commercial process. A special selected strain of *Aspergillus oryzæ* is grown on wheat bran. Everything is sterilized so that the growth of other fungi will not disturb the production of diastase, and the mold is fed artificial chemical compounds of nitrogen, phosphorus, and potassium to secure a maximum mycelial mat on the carbohydrate used. A culture of this sort produces unusually strong diastase and hence is called "taka," meaning "strong" in Greek or "high" in Japanese.

Wheat bran is used as a substratum since it is rich in albuminoids and phosphates, and being of a loose, coarse structure allows a maximum growth of the fungus. The bran is steamed, cooled, and the fungus added. In order to insure an abundance of air, the bran is spread out in thin layers about 1 to 1½ inches in thickness in the incubating room which is kept at a temperature of about 25°C. After 24 hours, the growth has become visible and after 48 hours it has attained its maximum diastatic powers. It can now be dried and used as a malt, or the enzyme can be dissolved out by water and the solution used, or, the solution can be evaporated in a vacuum pan to a thick syrup.

The method used in isolating the enzyme from the solution is to take a solution containing about 18 to 20 per cent of the extractive matter and to add, slowly, four to

five times its volume of strong alcohol, with shaking. A flocculent precipitate is formed. The alcohol may now be decanted off and the precipitate centrifuged out. The thick paste which comes from the centrifuge is washed with alcohol to dehydrate it and is then put under a hydraulic press to squeeze out the alcohol. It is then air-dried and powdered to make the commercial diastase.

Taka-diastase will convert one-hundred times its weight of dry starch into sugar in 10 minutes, but differs in some ways from the color which malt diastase produces with Lintner's guaiacum hydrogen peroxide, only a cloudy white color appearing; although this test may not be a test for diastase but only for some of the substances associated with barley diastase.

Taka-diastase differs also in the changes which it produces in a starch paste from the malt type of enzyme. If a very large quantity of starch paste is divided into two portions, to one of which malt diastase is added and to the other, Taka-diastase, it will be found that while the amount of sugar may be the same in either case, the Taka-diastase portion will be clearer and more liquid. This suggests that there are two enzymes in Taka-diastase, one a starch-liquifying enzyme and the other a sugar-forming enzyme.

Later investigations have shown, in fact, that Taka-diastase contains at least five other enzymes acting on phosphate, lecithin, sulphate, inulin and cellulose.[58]

Aspergillus niger also produces diastase but not in the quantity formed by *A. oryzæ*. Went[77] found that the former fungus produced diastase in quantity for a few days after its spores germinated, but that it lost this capacity after 5 days and the enzyme seemed to disappear. Very little could be found in the culture medium, but whether this meant that the enzyme was intracellular, or destroyed, could not be determined.

Other Fungus Enzymes.—*Aspergillus niger* and some green Penicilliums split sugars by the action of numerous enzymes, among which have been demonstrated invertase, maltase, threhalase, and raffinase. Enzymatic splitting of disaccharoses has long been known but the breaking up of a trisaccharose, melectricose, into dextrose and a disaccharose, analogous to maltose, has also been demonstrated.[35]

Some enzymes are secreted into the surrounding liquid while others are held in the cells of the fungus, *intracellular*, never passing outside the cell walls. Cell sap pressed out of the mycelium of *A. niger* and added to a solution of glucose will convert the glucose into alcohol in sufficient quantities to give the iodoform test.[37] This enzyme has been much more thoroughly studied in the yeasts.

Newcombe[59] isolated and demonstrated an enzyme of fungus origin which would dissolve the cellulose wall of the barley. This enzyme, which he named *cytase*, was subsequently renamed *cellulase*, since it changes cellulose.

The walls of the cells of the plant upon which Rhizopus is sometimes parasitic, such as the potato or sweet potato, become a softened mass of material by enzyme action.[76] The production of this enzyme (*pectinase*) occurs at all temperatures but with a maximum at 9°C. The degree of parasitism has nothing to do with the amount produced. If thin slices of sweet potato are placed in a solution in which enzymes from the fungus have been already developed by the growth of the mold mycelium, they will gradually soften although no mycelium is present, the enzyme being *extracellular*. Even the spores of *Rhizopus tritici* contain enzyme and the solution from germinating ones is capable of separating the cells in slices of sweet potato.

The Aspergillaceæ probably are all able to digest proteins by the action of proteoclastic enzymes. *A. niger* growing on ground peas will decompose the proteins and amino acids, such as tyrosin, with leucine, arginine, and lysine formed. *A. flavus*, one of the commonest of saprophytes, is able to digest albuminoids and starch.[41]

Penicillium camemberti, the organism which ripens Camembert cheese, secretes a variety of enzymes to bring about these changes. Dox[19] grew the mold in mass on a

liquid medium from which was separated a total of ten enzymes, among them being enzymes which would dissolve almost any of the nitrogen and carbohydrate combinations likely to be found in cheese. The fungi are capable of attacking almost any of the ordinary natural carbon compounds.

Are the enzymes always present in the fungus cells (spores and conidia) or is some special stimulus necessary for their production? Kopeloff[40] claims that no stimulation is necessary to produce enzymes (such as invertase) in the spores of the common fungi, such as *A. niger* and *P. expansum*, enzymes being already present in them, as may be demonstrated by heating them for 30 minutes at 63°C., killing the cell but not the enclosed enzyme. By adding some of the enzymes, after heating, to a solution of cane sugar they will invert some of it in 3 hours. Rhizopus also, as already noted, seems to store pectinase in its spores.[76]

The enzymes are much more resistant than the cells which produce them and probably persist long after the cells have ceased to function. No data on the longevity of the enzymes of a mold are available but Miehe[47] made the interesting observation that the diastase of barley will keep for many years. Some rye grains which were certainly 112 years old and which may have been kept for 280 years, contained a substance, which, on extraction, would dissolve starch, although the grain had long lost its ability to germinate. On the other hand, emmer from tombs of the eighteenth dynasty in Egypt gave no trace of diastatic ferments.

2. The Germination of the Spores and Early Growth of the Mycelium of Penicillium and Aspergillus.—The ease with which these spores of mold fungi can be germinated has caused them to be favorite objects of physiological experimentation. The great resistance of fungus spores to extreme low temperatures has already been noted in the experiments of Becquerel[3] who exposed the dried spores of various fungi to temperatures of 180 and 253°C. without destroying their powers of germination.

The mycelium of the molds is readily killed at temperatures much below the boiling point of water, probably for most of them, between 50 to 60°C. The spores are much more resistant since their protoplasm contains much less water, and the death of protoplasm is the result of the coagulation of the proteins. There is probably considerable variation in the resistance of different varieties, but cultures of *A. flavus* will continue to grow after an exposure for 30 to 60 minutes to a temperature of 45 to 65°C. The ordinary pasteurization process, in which the milk is held at 62.8°C. for 30 minutes, will kill practically all the mold spores, so their resistance to heat is not great.[72]

Aspergillus oryzæ spores retain much of their germinative power even after many months. An experiment on spores which had been kept 7 months, 2 years, and 4 years showed that, while the percentage of germination diminished with age, many conidia of all ages were still viable in sealed tubes; spores of *A. oryzæ* and *R. nigricans* still grew after 12 years.[46,36]

Cramer's Explanation of Spore Resistance.—Cramer[15] attributed the resistance of the spores of *Penicillium glaucum*, to desiccation and coagulation by heat, to the large percentage of dry substance which they include, as shown in their analysis:

	Percentage
Nitrogenous material (proteins)	28.44
Ether extract (fats)	7.34
Alcohol extract	30.46
Starch	17.00
Cellulose	11.13
Ash	1.91
Undetermined	3.72
	100.00

The so-called "starch," a carbohydrate (possibly glycogen), may be converted by acids into invert sugar. He considers the spores of the fungus to have cores of very concentrated protein with cell walls of cellulose- and carbohydrate-like compounds. The outer cytoplasm is filled with carbohydrate-like bodies which are soluble in alcohol and very hygroscopic (Fig. 59). The resistance to dry heat is due to the fact that these hygroscopic bodies absorb the water from the proteins; the resistance to steam is the result of the absorption by the hygroscopic bodies of any water which might penetrate the cell wall and it is only after they have become saturated that the proteins begin to take up water. The small amount of water in the proteins protects them from coagulation by heat whether wet or dry.

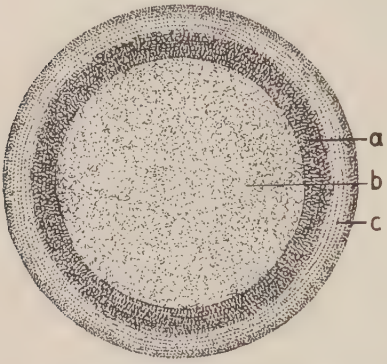

Fig. 59.—Spore of mold or bacterium according to the Cramer theory of the arrangement of materials: *a*, dense inner coating impregnated with a carbohydrate which takes up water; *b*, very dense protein; *c*, true cellulose wall.

Acidity and Spore Germination.—The spores of fungi such as *A. niger*, *P. cyclopium*, etc., germinate best in a slightly acid medium although germination is good from neutral pH7 to a pH of 3.1 to 2.8. Below the latter acidity, the germination is inhibited.[75]

The Amount of Water Necessary for Germination.—The molding of bread from the growth of the green Penicilliums or Aspergilli is one of the commonest occurrences of the summer months when it is kept wrapped in waxed paper to retain its moisture. The moist air inside the paraffined wrapper is sufficient to enable the mold spores to germinate in contact with the damp bread. The spores of Penicillium and Aspergillus germinate in air with an humidity as low as 82 to 84 per cent.[43] Since the air in the middle of the summer sometimes attains such a degree of humidity, it might be possible for the spores of these fungi to develope germ tubes even if they did not happen to be in a drop of water. If conditions should then become drier, the fungus might still continue to survive, if the germ tube were in contact with the moist bread and surrounded by a fairly well-saturated atmosphere. If a mold germ tube, therefore, did not find the necessary water or food at once, it could often get enough water from the air or from the relatively moist substratum to keep it alive. The spores of *Aspergillus flavus* after germinating in bean decoction for 8 hours will, after being filtered and washed with distilled water, still survive (almost 100 per cent of them) in it for 80 days.[20]

Effect of Traces of Food Substances on Spore Germination.—While the spores of the molds germinate in distilled water, they do much better in water in which some substance, even the smallest trace, is in solution. A trace of ether or alcohol increases the germination of *Aspergillus flavus* spores and germination is better in water which has stood for a long time in an open, paraffin-lined flask absorbing various gases from

the air of the laboratory. Even a little vaseline from a sealed slide increases[20] the percentage of germination 10 to 20 per cent.

The marked effect of minute quantities of food materials on the germination of spores is further emphasized by experiments which show that the volatile substances which arise from the leaves and fruits of apple (the volatile acids which produce the so-called *aroma*), also the fragrant leaves of the rue and Eucalyptus, increase the percentage of germ tubes of *Botrytis cinerea*, a saprophytic (occasionally parasitic) mold.[12]

The petals of various flowers exosmose sufficient substance into distilled water to give a stimulus to spore germination. The petals must be of a type which wet readily or the effect cannot be noticed. Brown's method was to crush up the leaves of fruit foliage of the apple or other plants to be tested, place them in a Petri dish together with some filter paper moistened with water, and then set ordinary slides, with the spores to be tested in a drop of distilled water, over the crushed leaves, being sure that one of the juices found its way into the drop of water. Other slides, for comparison, were placed in similar dampened Petri dishes without the crushed leaves.[13]

Mold Mycelium Has a High Osmotic Pressure.—The spores and the germ tubes which come from the spores of molds such as *P. glaucum* and *A. niger* are very difficult to plasmolyze with salt or sugar solutions. The osmotic pressure inside the mycelium, and especially in the spore, maintains turgor in spite of a high percentage of salt or sugar in the medium around them. Osmotic pressure in their cell sap is sometimes as high as 250 atmospheres for Penicillium and *A. niger*, when they are grown on very strong solutions of sugar.[60]

In parasitic fungi this osmotic pressure is much higher than that of plants or fruits, such as apples or strawberries, upon which they grow and from whose sap they withdraw soluble materials.[33] The soluble material which produces this enormous pressure inside them does not seem to have been investigated. In the higher plants, the osmotic pressure is a combination of that obtained from the soluble salts and carbohydrates, the latter mostly sugars of various types. The former is fairly constant, while the latter varies at different times of the day and in different weathers for plants in a single habitat, but the total pressure is very high only in desert plants and in those which are able to live in fairly dry soil. The osmotic pressure of desert plants never rises above 100 atmospheres,[26] so that the molds must be considered as one of the types adapted to growth in habitats where the substratum has a minimum of available moisture. The ability of the molds to adapt themselves to such locations means that while the food may be abundant and may have a strong concentration, it can still be absorbed because the mold mycelium has a still higher osmotic pressure.

Sugar or Salt in Culture Solutions.—Sugar develops a high osmotic pressure when in solution and retards development due to this property alone. Salt, on the other hand, not only produces a high osmotic pressure but is also a mild preservative, the Cl ion being slightly toxic. On jellies, jams, preserves, and syrups with 60 to 70 per cent of cane sugar, mold growth is very slow.

Common salt, even in small percentages, retards the growth of hardy molds, such as Aspergillus. Molliard[48] found that as low as 1 per cent would retard the formation of conidia; 2 to 5 per cent would slow up growth; 10 per cent would almost stop it; and 12 per cent would check it entirely. The suppression of conidia seemed to be due to the formation of HNO_3 in the more concentrated salt solutions.

In a combination of sugar and salt in a nutrient solution, the question arises: Can the mold make use of the sugar if considerable salt is present? Haenseler[30] tried *A. niger* on various combinations of salt and sugar in the medium in order to answer this question, using salt solutions with a pressure of 0.5, 2.1, and 4.2 atmospheres, with the same amounts of sugar in each. In other experiments, the same salt solution

was used in each but the amount of sugar was varied. The yield obtained from the cultures that had attained a maximum growth indicated that abundant nitrogen neutralizes the toxic effect of the salt and that, with the same concentrations of salt, growth depends on the amount of sugar added to the solution.

Acidity Helps Mold Growth.—Not only is spore germination improved by conditions of active acidity, the majority of the fungi attaining a maximum spore germination with a pH content of about 3.0 to 4.0, but the mold protoplasm itself seems to be acid in its reaction,[75] the mycelium of *Rhizopus nigricans*, with a pH of 5, indicating a rather high acidity. The function of the acid may be to improve the absorption of food materials from the substratum. The method used for obtaining this reaction[66] was to wash the mycelium for a few seconds to a minute with eosin, methylene blue, or safranin and then with a buffer solution of 1 mol of phosphoric acid plus 0.1 mol of NaOH, the color reaction being brought out by the latter solution.

Reaction of Medium and Color of Colony.—The reaction of the medium on which a mold is grown may affect the color of the colonies grown on it. Thom and Church[73] obtained green colonies in members of the *A. flavus* group when grown on acid media, the color being still further intensified when they were exposed to the fumes of hydrochloric acid but changed to a buff over ammonia fumes.

The color changes which fungus cultures undergo as they age are due to an increase in the number of conidia and to the thickening of the conidial walls by spines and emergences. Acidity and alkalinity in the culture medium do not at once affect the color of a mold such as *A. flavus*, as it has considerable independence of reaction but, with this mold, a bright green color is characteristic of an acid reaction and a dull brown of an alkaline one. The color is partly in the wall, a yellow and then a yellow-brown, and partly in the plasma, a light green or blue-green. The wall is colored darker brown by alkalies. *A. glaucus* mycelium has, in various strains, at least four different coloring matters in various proportions: (1) a yellow, fluorescent, (2) a wine-red, (3) a brown, and (4) a violet. The chemical composition of these coloring matters is very complex.[5]

The Poisoning of Molds by Chemicals.—While the presence of traces of foodstuffs is so essential to germination, the addition of even the slightest traces of the salts of some of the heavy metals such as mercury, copper, silver, etc., will check germination and, if in quantity, will kill the spores so that they will not germinate. Nägeli[57] observed that he could not grow certain species of algæ in water which had been distilled in a still with a copper coil, and that he could kill Spirogyra by placing a well-scoured German penny in the jar in which it was growing. Enough of the copper passes into the water to destroy the algæ. He found the same sensitiveness in the same species of fungi although many common molds are fairly resistant. The bacteria are also sensitive to the presence of these mineral salts. Nägeli was so impressed by the greatness of the effect and the smallness of the chemical producing it that he coined a new name for the phenomenon, *oligodynamic* (oligo = much, dynamic = force). The control of the molds and of many fungus diseases of plants is based on the fact that the fungus is much more susceptible than the host plant to the action of the salts of certain minerals, especially copper and mercury. The most effective substances in killing the spores or in preventing their germination[14] are mercuric chloride and silver nitrate,

either of which is poisonous even in dilutions of one part in a million. Next in value are potassium dichromate, potassium chromate, and formaldehyde. The salts of nickel, cobalt, iron, copper, and zinc are poisonous in about the order named. Hydrocyanic acid, a substance which is deadly poison to animals, is only fairly poisonous to plants; while strychnine, another poison for animals, does not greatly affect plants. The silver and mercury salts unite with the proteins of the spores or hyphæ to cause coagulation, and the secretion of certain internal enzymes may cease, since some of them (diastase) are known to be very susceptible to metals such as copper.[23]

Poisons May Be Adsorbed by Small Solid Particles.—The efficiency of some of these poisons is markedly reduced in the presence of a finely divided foreign substance, insoluble in the media in which the cultures are growing.[25] Aspergillus or Penicillium growing in a beet decoction, to which copper sulphate or sulphuric acid has been added in very small amounts, has the toxicity of the chemicals much reduced if finely broken glass or filter paper is added to culture. These relatively insoluble substances remove the poisons from the culture media by the physical process known to the chemist as *adsorption*. In some way the poisonous molecules or ions attach themselves to the surfaces of these particles of sand or filter paper and are removed from the solution, the attachment being physical rather than chemical.

3. The Availability of Different Forms of Carbon as the Source of Energy.—The fungi can use any of the ordinary starches and sugars either under natural conditions or in artificial cultures, although a number of them must be changed chemically by enzyme action before they are assimilable. An enzyme is secreted to bring about such chemical conversion so that the fungus, almost regardless of the substratum upon which the spore may fall, can make available and use any organic carbon compound. Some of them are much more assimilable than others and require much less energy to reduce to a form where they can be built into fungus protoplasm or oxidized as a source of energy. The energy used for this purpose by the yeasts and molds is much greater than that used by the same weight of green plants, and, undoubtedly, a large part of it goes to the rapid conversion of the food substances into an easily available form. A mold grown in a solution of glycerine and ammonium sulphate will increase in weight one thousand times in 20 days. It is questionable if any green plant could duplicate such rapid growth.[8]

The general method used for determining the value of various food constituents for the fungi is to grow them in pure cultures with a basal liquid medium to which various types of carbon or nitrogen are added. This basal medium contains all the salts necessary for growth but lacks either carbon or nitrogen. The basic solution is the constant; the carbon or the nitrogen is the variable. The growth is allowed to go on until

the mycelium seems to have attained its maximum weight, when it is removed from the flasks, dried, and weighed. The remaining liquid in the flask can be analyzed to determine how much has been removed by the growth. The method would seem to be simple and accurate but, unfortunately, complications are introduced by the fungi themselves in their growth. The splitting up of compounds, such as ammonium sulphate, leads to the production of sulphuric acid, or, if the culture is allowed to grow too long, some of the mycelium may die and the dead material be used as a source of food by the remaining living hyphæ.

Two strains of the same fungus very rarely have exactly the same physiological requirements and much of this work has been done by physiologists and chemists who made no attempt to determine the name of the fungus with which they were experimenting. If it was a green mold, it was *Penicillium glaucum* or *Aspergillus glaucus* regardless of the numerous species which went by these names.

The Availability of Various Carbon Compounds.—Nevertheless, from this experimentation, some facts have been learned as to the ease with which fungi can make use of the various carbon and nitrogen compounds. One of the best early investigations on the availability of carbon compounds was by Nägeli[56] who arranged them in the following series: (1) sugar, (2) mannite, glycerine, leucine, (3) tartaric acid, citric acid, succinic acid, asparagin, (4) acetic acid, ethyl alcohol, quinic acid, (5) benzoic acid, salicilic acid, propylamine, (6) methylamine, phenol. Nägeli claimed that the carbon had to be grouped properly in the molecule in order to be assimilated, but his hypotheses have been almost entirely disproved.

Various other later lists have been made and the availability of particular sugars and alcohols has been studied.

Glucose is the most readily available substance and is used by the fungi as a source of carbon in preference to any other chemical. It can be removed from a mixed sugar and the others left. With saccharose in a culture solution as the source of carbon and *Aspergillus niger* as the fungus, the saccharose is inverted into dextrose and levulose; all the dextrose is destroyed but only one sixth of the levulose. The fungus apparently will absorb levulose only when dextrose is not plentiful.[48]

Glucose has been considered the sugar of respiration, levulose, that of tissue building, but with both present in a culture solution or in an acid solution of sucrose, Brannon[10] was unable to find very much difference in the rate of use of the two sugars, and levulose can be used as readily as dextrose for respiration.

Any readily oxidizable source of carbon such as alcohol, acetic acid or the acetates will be absorbed and used.[32] On the other hand, while ethyl alcohol will permit the growth of the mold, none of the cultures containing it develop spores. The reason for this lack of availability when the fungus comes to spore production is not clear. The molds will grow in ethyl alcohol[8] but not in methyl alcohol, and alcohols such as propyl, isopropyl, and amyl are poisonous, although isobutyl will permit some growth if mineral salts are present. Glycerine, ethyleneglycol, erythrite, mannite, and dulcite are all good sources of carbon while phenol, cresol, resorcin, and phloroglucin are either poisonous or poor sources.

The amount of carbon in the carbon dioxide produced in respiration approximates, added to the carbon built into the mold body, the carbon derived from the sugar consumed. Of the energy which is obtained in the form of carbon compounds by

A. niger, approximately 70 per cent is utilized in growth, and the other 30 per cent in metabolic processes or given off as heat.[71] Some of the carbon is stored in partially oxidized compounds such as oxalic and citric acids.[22,50] This use of carbon in the upbuilding of the plant body, and as stored compounds inside or outside it, means that the ratio of the carbon dioxide formed to the sugar used is always a fraction.[31]

4. The Fixation of the Nitrogen of the Air by Fungi.—It was at one time believed that many of the molds assimilated a part of their nitrogen from the air and were not entirely dependent upon the media on which they were grown for nitrogen compounds. Such a view has been disproved and it is doubtful if any of them can utilize atmospheric nitrogen for building proteins.

Duggar and Davis[21] decided from tests made on nitrogen fixation by *Aspergillus niger*, *Macrosporium commune*, *Penicillium digitatum*, *P. expansum*, and *Glomerella gossypii* that fixation, if present at all (with one exception), was in such small amounts as to be almost negligible. Previous work had been contradictory, although the weight of the evidence was much against any fixation of nitrogen by any of the common fungi found growing saprophytically in the soil. Since a large part of the destruction of organic matter in the soil is carried on by fungi, they might play an important part in fixing atmospheric nitrogen if they were capable of doing so. Since they cannot fix nitrogen, however, they are to be regarded only as agents of destruction for the carbon compounds, not as increasers of the nitrogen in the soil.

5. The Best Source of Nitrogen from the Substratum.—The nitrogen in the mold protoplasm is largely as combined amino acids. The question now arises, *Is it better to supply the nitrogen of the media in the form of similar amino acids or as ammonium or nitrate combinations?* Are complex chemicals or simpler ones the better? The answer involves the whole matter of the absorption of materials.

The Amino-acid Theory.—Czapek,[16] after using a long series of compounds of nitrogen, decided that the best compounds for the maximum growth of the fungus upon which he was working, *Aspergillus niger*, were those which contained the amino acids. The amino acids are a preliminary stage to the formation of fungus protoplasm and if the food is available in this form, the fungus does not have to use energy in transforming it into intermediate products. Asparagin was the amino acid best suited to the purpose, his conclusion following the Pfeffer hypothesis that, in the higher plants, the protein compounds are synthesized from asparagin and sugar by a process of condensation.[17,18]

The Nitrate or Ammonia Theory.—Other experimenters with the nitrogen compounds consider that the absorption and the resulting growth are better if they are in the form of ammonium compounds or nitrates, since the amino acids must be reduced to this condition before they can be used. From simple nitrate compounds the fungus is able to build the same complicated proteins that are to be found in higher plants. *Aspergillus niger* can form its protein compounds from potassium nitrate as the source of nitrogen, the mold mycelium containing many of the amino acids found in all plants.[1] Ritter[65] found that ammonium combinations are, in general, the best media for fungi but that they should be combined with weak acids.

Some fungi do better on nitrates while others, such as *Aspergillus niger* and *Rhizopus nigricans*, assimilate nitrates poorly and must be fed with ammonium compounds.

Brenner,[11] using *Aspergillus niger* as the test fungus, tried a series of compounds of nitrogen and found that, in general, ammonium compounds are better than nitrate compounds but that the fungus cannot use nitrites.

Cultures change in reaction due to the absorption of certain ions and the retention in the medium of the ions with which they had been combined. *A. repens* will not use the ammonium salts of strong acids, since the utilization of the ammonia sets free the mineral acid ions, rendering the solution highly acid, but it will use much better the ammonium salts of weak acids as the ammonia from them can be absorbed without these acid and toxic results.[2]

Autolysis.—All of this work on nitrates has been subjected to severe criticism by Boas[6] who points out that the fungus growth releases, from many of the compounds, acids such as HCl and H_2SO_4 which may be injurious to further assimilation, and that the weight of the fungus mat does not represent the real value of the compound as a source of nitrogen. The long period of growth of fungus cultures before they are weighed gives an opportunity for a self-digestion of the fungus, *autolysis* (auto = self, lysis = setting free or destruction). In order to determine when autolysis started, Boas made a number of successive weighings to get a maximum growth. By using carbon compounds, such as glycerine, malic acid, and quinic acid, instead of the sugars, ammonium sulphate and ammonium phosphate were found to be better sources of nitrogen than asparagin, which had been found so favorable by Czapek. The amino acids have first to be deaminized before they can be utilized by the fungus, and this process requires energy so that ammonium salts are the most favorable for growth, being much superior to the peptones and acid amides.[7]

Klotz,[38] continuing the study of the relative merits of the nitrogenous combinations with special reference to the autolysis which occurs in the cultures, believes that it does not set in until the carbon compounds are exhausted in the media, when the fungus attacks the peptone in the cultures themselves in order to get the carbon. Autolysis can be determined in a culture by a decrease in weight after the fungus has attained its maximum growth and by the presence of ammonia in peptone and potassium nitrate media.

General Conclusions.—The general conclusions to be derived from these studies on nitrogen nutrition seem to indicate (1) that the various species vary in their requirements; (2) that the formation of various products in the used culture media may check growth, these substances being the result of the fungus activity; (3) that the fungus will begin to digest itself when food becomes unavailable in the media.

6. Effect of Used Culture Media on Fungus Growth.—Fungi do not grow as well on media on which fungi have been previously grown, although food material is still abundant. In growth, fungi secrete into the culture solution or break down some of the constituents into substances which are deleterious to growth. Such a used medium is known to the English as a "staled solution." Substances restricting growth are combined with calcium in higher plants to form insoluble compounds, which are deposited, as crystals or other bodies, in cells set apart for their disposal. Fungi and bacteria, growing in culture media, simply throw them off into the substratum where they later serve as a poison. In parasitic species, these waste products are often the cause of the death of the host cells and, later, of the fungi themselves.

The chemicals produced in fungal growth may be very simple ones, such as alkalies which render the medium alkaline, or they may be complex proteins which may be coagulated by heat and then filtered out.

The negative reaction of fungi to their own products has already been noted in the chemotropism of the hyphæ and germ tubes of *Rhizopus nigricans* where growth is always away from other hyphæ. This reaction enables the fungus to find new regions where food is unexhausted and excretion products are not abundant. It is this property which enables a fungus to spread inside a host plant, avoiding regions already preempted.

In cultures of *Aspergillus niger*, with urea as the source of nitrogen, the solution soon becomes quite strongly alkaline.[6] The fungus is unable to stop the production of urease and continues to split up urea into alkaline compounds to its own detriment, making the medium alkaline. Cultures of *Botrytis cinerea* and of *Oidium* would not do this.

Even while food is still abundant in old, used, culture media, the reaction may be changed and certain products formed which injure growth. These substances seem to be ammonia and various proteins. The former may be removed by correcting the reaction so as to render the medium acid, and the latter, by boiling, followed by filtration. These products are not specific against the fungus forming them, as the growth of one fungus may render the culture medium toxic to all others.[9,63]

The effect of the ammonia evolved in fungal growth[12] can be shown by exposing inoculated Petri dishes, filled with media, over old cultures of *Sphaeropsis malorum* for two days:

Growth in 2 days of 11 colonies, over old colonies	.42 centimeters, average
Growth in 2 days of 12 colonies, control	1.57 centimeters

Fusarium solani and *Aspergillus niger* left substances in the culture solution which were helpful.

Some fungi, such as *Botrytis*, are non-staling but others such as *Fusarium* produce ammonia in quantity and are affected by it. Since *Botrytis* produces no staling products, it is a fungus of unlimited growth, while the Fusarium's growth is limited by the by-products which it produces, and the colonies attain only a certain size.

7. The Formation of Rings in Mold Colonies.—Old cultures of many of the molds show clearly marked concentric rings in the colonies, a zone of rather thin mycelium alternating with a zone of densely fruiting mold growth, so that in a large colony of Penicillium, six to eight, or even more, zones can be distinguished. The zonation is purely a growth process, but the stimulus by which the alternate rings are formed is not quite clear.

The Rings Are Due to Alternating Light and Darkness.—Hedgcock,[34] observing them on cultures of Cephalothecium, came to the conclusion that the sparse rings of growth were formed in the daytime and the denser ones at night, daylight inhibiting the growth by its blue rays. By using various sources of light, he was convinced that under red and orange light the fungus would form only a mass of spore-bearing hyphæ over its whole surface, uniform in growth. Molz[52] was of the same opinion from observations made in the open on the rotting of apples by the ordinary brown rot and from laboratory cultures of the fungus. Galemaerts[28] exposed cultures of Aspergillus, Penicillium, Cephalothecium, and other fungi to the action of the constant light

from a Cooper-Hewitt light, while the temperature was maintained constant, and found, that, as contrasted with the control cultures which were kept in the dark, the light hindered spore formation and that the zonation was undoubtedly the result of the change from light to darkness. Temperature had nothing to do with the process; the ultra-violet rays of the lamp were the real stimuli. Bisby,[4] however, found the zones in *Fusarium discolor sulphureum* to result either from variations in light or temperature, light affecting the tips of the hyphæ and preventing normal growth.

Zonation is the Result of Other Stimuli.—While the results obtained by these investigators would seem to be conclusive, just about as many hold other reasons than light and darkness to be the causes of zonation.

Stevens and Hall[67] take the position that alternating light and darkness have nothing to do with zonation; that it is the result of the growth of alternately thick and

Fig. 60.—At right, plate culture of *Ascochyta chrysanthemi* showing that formation of zones is not coincident with the diurnal changes, days indicated by ink circles. At left, theoretical explanation of formation of such zones by alternating thick and thin layers of mycelium. (*From Stevens and Hall.*)

thin mycelial strands. The hyphæ branch and rebranch in order to avoid each other, growing out in a radiating manner for this purpose. When the growth becomes too dense for the best development of the fungus, ruiting occurs and only a small percentage of the hyphæ are able to continue to push outward from the common center. A zone of growth where the hyphal strands are far apart will result, but, as the branching and outward growth are repeated, a dense zone develops. By comparing the growth with the alternation of days and nights, they were able to show that the two were not at all related (Fig. 60). Fruiting seems to occur in this dense, crowded zone but is secondary and dependent on this alternation of mycelial growth. It may result in a conidial development among the denser hyphæ, making zonation all the more conspicuous or in the formation of circles or sclerotia (Fig. 61). Reidemeister[64] incubated piled up Petri dish cultures of *Botrytis* in such a manner that they were rather strongly illuminated from one side. On the sides turned toward the light, the sclerotia, which this fungus produces abundantly, were uniformly diffused over the surface, while on the shaded side they were in the rings. The sclerotia were

diffused in a similar way over plates exposed to the direct rays of the sun in a greenhouse. The variation in light, therefore, would seem to have little influence on the formation of rings of sclerotia.

Munk[53] has also severely criticised the suggestion that day and night cause these alternating regions.

Zonation and the Liesegang Rings.—A third suggestion is that the zonation phenomena are to be placed among the so-called "Liesegang rings."[44] Various chemicals such as NaCl dissolved, in colloids (gelatin), when precipitated out of solution by another chemical, such as $AgNO_3$, form zoned rings of precipitate. The chemical action is rythmical. Such chemical rings are very analagous in appearance to the zonation in fungus cultures.

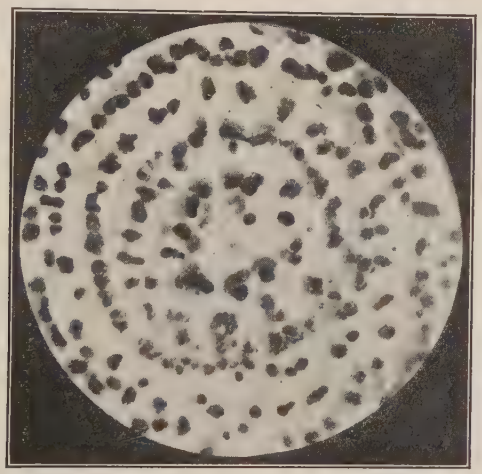

FIG. 61.—Zonal formation of sclerotia in *Sclerotinia Libertiana*. (*After Stevens and Hall.*)

The question of zonation must be left unanswered at present, since the evidence is almost equal as to the effect or lack of effect of light. The fact may be pointed out, however, that fruiting in fungi arises from some unknown stimulus, perhaps from an attempt on the part of the fungus to free itself from some chemicals which are injurious to further growth, and that, as these chemicals are deposited in the spores, the fungus takes a new start, undergoes a process of rejuvenation, the hyphæ in the thin zone being, therefore, young hyphæ.

The Effect of Light on the Fungi.—The general statement is sometimes made that light injures mold growth and that molds do not thrive as well in a well-lighted room as they would in a dark chamber. Well-conducted and convincing experiments on the subject of light effects do not seem to be abundant, but any checking of growth may be due to increased respiration.

Kolkwitz,[39] using cultures of Mucor, Penicillium, *Aspergillus niger*, *Bacillus vulgare*, *Bacillus prodigiosus*, and *Oidium lactis*, illuminated half of them with an electric incandescent light and found that the illuminated cultures showed, in general,

an increased respiration of about 10 per cent. Maximow[45] investigated, with elaborate care, the effect of the light from a 15-ampere electric filament on the respiration of *A. niger* growing on Raulin's solution. The culture dishes were sealed with paraffin and then placed (the uncovered control and the covered Petri dishes) inside a larger vessel in which water was in active motion. The covered dish naturally would be the warmer if the two were placed in the light, since the dark covering would absorb the light, but this method removed the extra heat, and the control and the dish in the light had the same temperature. The age of the culture had much to do with the part played by the light; young cultures were little influenced in their respiration by the light or darkness but in the old cultures, especially with the media becoming exhausted and food scanty, respiration was increased by the light. The influence of the light was greatest during the first 30 minutes and then gradually lost its force; alternating light and darkness lessened the effect of the stimulus. With young cultures of *Mucor stolonifer*, the increased respiration for the first ½ hour seemed to have an injurious effect on the later development of the fungus.

So far as shown, strong light increases respiration and this increased respiration is not a real benefit to the later growth of the mold. Only general statements about the actual amount of mycelium produced after such treatments are available, but the speeding up of respiration apparently lessens the weight produced. In general, therefore, it may be said that strong light is injurious to mold growth, particularly when it falls on young plants. The water content of the fungi seems to have a more marked effect on respiration, however, than the light which falls on them, the very watery ones respiring the most.

Diffused Light.—Many molds have an optimum illumination, usually a diffused light, into which they will grow or the sporangia bend from their bases.[29]

Ultra-violet Light.—Exposures of cultures of *Mucor guillermondi* to ultra-violet light induced great quantities of sporangia in the zone of stimulation but in *Mucor genevensis* many zygospores were produced instead.[55] An exposure of cultures of *Glomerella cingulata* to the action of the ultra-violet (2,760 to 3,130 Ängstrom units) stimulated them to the production of conidia and induced the formation of sexual spores.[68,69]

8. Fungus Spores Are Spread by Moving Currents of Air.—The spores of Penicillium are almost as small as the minutest particles of dust and are readily carried into the air by the slightest disturbance.[27,24] The measure of value for the movement of such particles has been established as a current of air moving at the rate of a definite number of microns per second. The following table[24] gives the size of some of the Penicillium spores and the wind velocity in microns per second necessary to carry them into the air:

	Size, microns	Speed of fall, centimeters per second	Wind velocity necessary to spread them
Smallest dust particles	.....	.....	50
Smallest Penicillium spores	2 × 2	0.015	100 to 500
Average Penicillium spores	3 × 3	0.033	100 to 500
Large Penicillium spores	4.5 × 4.5	0.075	500 to 1,000

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CHAPTER XIII

THE CULTURE, REPRODUCTION, AND USES OF THE CULTIVATED OR DOMESTIC YEASTS

The yeasts are a very diverse group; the relationship of many of its members is in doubt, and some of the forms classed at one time as yeasts are undoubtedly only stages in the development of mycelium-forming species of fungi. The yeasts are of such practical importance that the grouping has been made, to some extent, on the basis or habitat or of physiological reactions that are of use to mankind and may express no real relationship.

Classes of Yeasts.—These microorganisms may be roughly divided into domestic yeasts and wild yeasts. The wild yeasts are further subdivided into color groups and morphological groups:

1. Gray wild yeasts.
2. Red or pink wild yeasts.
3. Black wild yeasts.
4. Mycodermas.

Some of the gray yeasts are known also as *cider yeasts* and *wine yeasts*, as they occur in the fermentation of these substances.

The old name *Torula* was applied by Pasteur to spherical types, often red or pink, but the designation *true yeasts* was reserved by Hansen for these species which form *endospores* inside the cells.

THE CULTIVATED OR DOMESTIC YEASTS

The ordinary yeasts used today in the household, the brewery, and the distillery were at one time wild yeasts which gave such excellent reactions of a particular type that they have been perpetuated in pure cultures, sometimes for many generations. They are divided into two classes according to the percentage of alcohol formed:

1. Distillers' yeasts capable of forming a high percentage of alcohol.
2. Brewers' yeasts with a lower percentage.

All are varieties of the yeast known as *Saccharomyces cerevisiæ* Hansen. The brewers' yeasts are divided by the writers on yeasts, such as Jörgensen[15] or Lindner,[17] into: (*a*) *top yeasts*, species which grow principally on the surface of the solution; (*b*) *bottom yeasts* found mostly at the bottom of the vats. The fame of certain breweries or even cities in Europe has been founded on the type of fermentation which some strains produce.

Some of the brewery yeasts bear the name of famous breweries, or cities, such as Pilsen, Frohberg, or Saaz, but many of them are only listed as numbers in the series of yeasts isolated by such noted fermentologists as Hansen, Will, or Lindner. No brewery would depend on the spontaneous fermentation induced by wild species, the whole process being controlled from start to finish with the greatest scientific precision in order to insure a uniform product with a characteristic quality.

THE DOMESTIC YEASTS

The type of yeast in use today for bread making in the United States is a distillery variety of *Saccharomyces cerevisiæ*, this race apparently producing the best bread. The description that follows, therefore, it will be understood, is based on the characters of the yeast found in the common bakers' yeasts, Fleischmann's and Magic Yeast (Yeast Foam), a plant of the high CO_2-producing variety. The old method of raising bread by a piece of leaven left from the last baking or the jug of yeast kept fermenting or obtained from the near-by brewery has been superceded by the purchase of these cheap and reliable substitutes. The methods by which these two types of yeast cultures are produced for commercial consumption by bakers and the general public are entirely different and it will be necessary to discuss them separately. The yeast cells in both cakes are in a dormant condition, particularly in the dry form.

Fleischmann's Compressed Yeast Cakes.—This type of yeast cake is monopolized by the Fleischmann Co. which makes it in 11 factories and is able, by an elaborate system of distribution, to get its product into the hands of the users before it is contaminated by mold growth.

The yeast is grown on the maltose sugar from rye and barley and is then mixed with cereal flours and molded into cakes (Fig. 62).

The ground barley and rye are mixed with barley malt and water. The diastase in the barley malt saccharifies the starch and converts it into malt sugar. The remains of the cereals are filtered out and the filtrate is a light-yellow, sweetish liquid known to the brewer as *wort*. This wort is acidified by allowing a culture of *B. delbrucki*, one of the strong lactic-acid forming bacteria, to grow in it. Into this acidified medium, a pure culture of the yeast is allowed to trickle, the yeast growing in it for some time, until a maximum amount is obtained. It is filtered, the yeast partially dried, mixed with cereal flour, and molded into cakes of about $\frac{1}{2}$ ounce each.

The yeast cake, as put on the market, contains, therefore, not only the yeast itself, but also a number of the acidifying bacilli and some cereal starch. The cake is rendered acid in order to check the growth of the putrefying bacteria and because the yeast plant flourishes best in an acid medium. The flour absorbs and retains some of the moisture from the yeast cells enabling the manufacturer to mold the cake into a permanent shape.

An examination of a cake of compressed yeast shows large starch grains and small, single-celled, almost colorless fungi, many of them with a smaller cell attached to one end or side (Fig. 63).

These cells may be roused from their quiescent condition by the addition of a nutrient solution in which they will grow. A large vacuole usually fills the center of

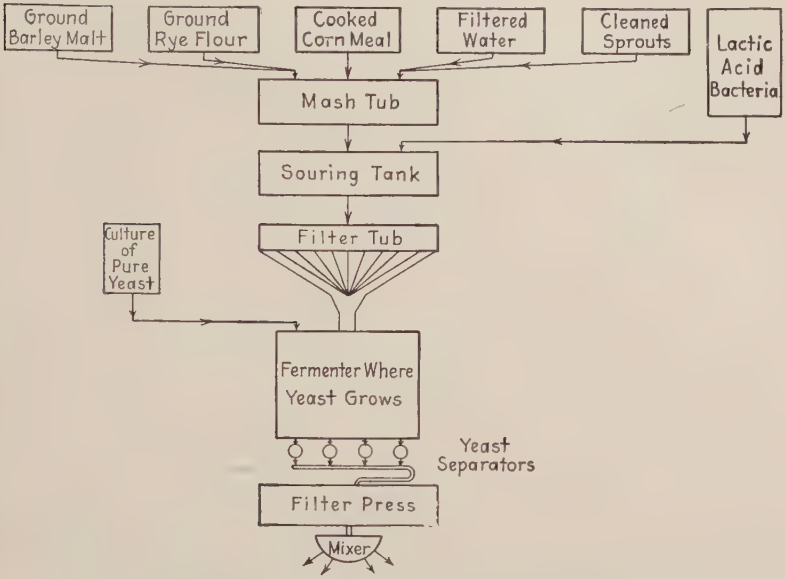

FIG. 62.—Diagram of plant for production of compressed yeast.

FIG. 63.—1, Yeast cells from cake of Magic yeast (dry); 2, starch grains from same cake; 3, wild yeasts from the cake; 4, yeast cells from cake of Fleischmann's; 5, starch grains; 6, *B. delbrückii*, lactic-acid producer in these cakes, × 600.

each of them. Growth has been checked by the dry condition of the cake and, as some of the cells were budding new cells as the cake was made, they are still to be seen in that stage.

Uses of the Lactic-acid Bacteria in the Cake.—The lactic-acid organisms present in the cake, together with the acid condition of the cake itself, restrain the growth of other bacteria. The only growths that are likely to appear on the cake, even after it has been kept a long time, are some of the molds or the yeast-like Mycodermas. The lactic-acid organisms maintain the acidity of the cake and give the yeast an opportunity to start growth in a slightly acid medium which is an advantage, as acidity promotes yeast growth. The lactic acid which these organisms produce gives some flavor to the bread but more is given by the yeast itself in its digestion of the gluten of the flour.

Yeast Cakes and Leaven.—The old-time breads were made by the help of impure yeast cultures or by the use of a piece of leaven. Leaven was a piece of dough saved from a previous baking which had been kept to start off again the new fermentation. When the leaven was added to the new lot of dough, its yeasts and other organisms started to grow and produce carbon dioxide and flavors, but since the organisms varied in this leaven, being dependent on accident, the bread produced varied in quality and flavor.

By the use of a single type of yeast with definite conditions for its growth, uniformity of flavor is maintained and, while it may not form the very fine flavor which was to be found at times in homemade bread, it is always the same and we have learned to like and expect a certain type.

Compressed-yeast cakes will not keep indefinitely even when held at a low temperature, as in a refrigerator, as mold or Mycoderma growth gradually appears on the outside, changing the reaction of the cakes and injuring the yeast cells. The length of time which a yeast cake can be kept is dependent on the temperature in which it is held but it should be good for 10 days in the refrigerator or 2 to 3 days at room temperature in the summer, the cake being usable as long as it is free from visible mold growth on the outside.

The Dry-yeast Cake.—The dry-yeast cake was the form in which the mothers of the past generation kept their yeast unless they happened to live in a brewery or distillery town where they could send and get a pitcher of yeast from the fermenting vats for use in bread making. While the life of the compressed-yeast cake is only 1 or 2 weeks, dry yeast will remain viable for months, under favorable conditions even a year. The keeping qualities of the dry-yeast cake make it available in regions where the compressed-yeast cake never could be taken. Dry-yeast manufacturers claim that the bread made from their cakes has a better flavor than that made from the compressed cakes. While the dry cake has the advantage that it can be kept for a long time, it has also the disadvantage that it must be started the day before baking, as the yeast cells are in a resting condition from which it is impossible to rouse them at once. An overnight soaking in a solution made of potato water or a weak sugar solution will again start them growing.

The method of production of the dry-yeast cakes is somewhat different from that of the compressed cakes. The yeast is grown in liquid medium composed of rye flour, malt, hops, and water. There is no filtering of the mash as it will all go into the cakes. When the culture is growing well, it is mixed with specially ground corn meal to form a dough. This corn meal has to be especially clean and free from fungi or the cakes will be contaminated from it. The dough is then cut into cakes which are placed on trays, and sent to the curing rooms where the yeast continues to develop while the moisture is being removed gradually from the cakes with their contained yeast cells. The whole process begins at the top of the factory with the ground grain; the yeast cakes come out of the ground floor in boxes ready for shipment.

The Use of Hops.—Hops are added to the yeast, as a preventative of the growth of bacteria, as they are to beer.[2] At the base of the bracts of the hop flower is formed a yellowish powder, known as *lupulin*, which contains the active principle, desired by the maker of yeast or the brewer, an essential oil giving the hop flavor and the resins which act as preservatives. These resins are at least three in number; two of them with a preservative effect in liquids to which they are added. They retard the growth of bacteria but not that of yeasts, giving the latter a chance to grow. As these resins become hard in old, dried hops, new hops are preferred, as the resins in them are still soft and soluble; many brewers store their hops at low temperatures.

The yeast cells in the dry cake diminish considerably in size in the drying process and undoubtedly many of them die (Fig. 63), but the slow drying probably preserves such a large percentage of them that an 8-hour soak, in water containing some food materials such as potato water with sugar, gives an actively growing culture.

Chemical Composition of the Yeast Plant

The ash of yeast contains:

	Percentage
Phosphorus pentoxide (P_2O_5)	54.5
Potassium oxide (P_2O)	36.5
Magnesium oxide (MgO)	5.2
Calcium oxide (CaO)	1.4
Silica (SiO)	1.2
Sodium oxide (Na_2O)	0.7
Sulphur trioxide (SO_3)	0.5
Chlorine (Cl)	trace
Iron (Fe)	trace
Total ash	100.00

An analysis of the yeast cake shows:

	Dry Substance	Moist
Protein	52.41	14.15
Fat	1.72	0.46
Glycogen	30.25	8.16
Cellulose, gum, etc	6.88	1.87
Ash	8.74	2.36
Moisture		73.00
Total	100.00	100.00

It is to be noted, however, that the percentage of glycogen given is that in a freshly made cake. By the time the cake is in the hands of the consumer, the glycogen content is only about half of this amount, the remainder having been used in respiration.

Glycogen in the Yeast Plant.—Glycogen ($(C_6H_{10})O_5)n$) is an interesting and characteristic carbohydrate found in animal and fungus cells. At certain stages of growth, it is very abundant in yeast cultures as a colloid in certain vacuoles or in the cytoplasm, but Clautriau[3] found its extraction in quantity from the yeast very difficult: (1) yeast cells rich in glycogen must be used, (2) the cells must be thoroughly broken up, (3) the gum and slime must be removed from the crushed cells. The best way to get a yeast rich in glycogen is to grow it in beer wort to which sugar has been added to make approximately a 12 per cent solution, or, to use sugar-beet juice which contains about the same amount of sugar. At 28°C., the growth is very active and the

plants fill in a short time with glycogen. The cells are very difficult to break but it is possible by grinding them in a mixture of precipitated silicate and calcium carbonate.

The glycogen obtained has the chemical formula $X(C_6H_{10}O_5) + H_2O$. It is split by diastase into maltose. The chemical properties of the glycogen of animal origin are similar but yeast glycogen is colored *brown-red*, in a solution of iodine, instead of the *brown-violet* obtained with animal glycogen. An increase in the temperature will take away the intensity of color in animal glycogen so that at 58 to 60°C. it entirely disappears, while with the yeast type 72 to 73°C. is necessary to bleach it. It may be possible that there are a number of different varieties of glycogen some of which are polymers.[5]

THE USES OF THE YEASTS

Bread Making.—The use of ordinary yeast to raise dough by the formation of bubbles of carbon dioxide scattered through it, is one of the oldest known household arts. In Biblical times it was known as "leaven" and was only a bit of the dough containing the yeast organism. The use of yeast for carbon-dioxide production is somewhat wasteful, as it involves also the formation and loss of a considerable quantity of alcohol, since it passes off during the baking of the bread. Liebig estimated this loss at 2 per cent of the food value of the bread and calculated that there disappeared from the ovens of London 300,000 gallons of alcohol in one year, and that enough toll was taken in Germany by the yeast plants to feed an additional population of 400,000 people.

Various other methods for making light dough have been suggested; the most practical is that of the Aerated Bread Company of London in which the flour is mixed with cold water strongly impregnated with carbon dioxide, the operation taking place in enclosed vessels where the carbon dioxide can be kept under pressure. After mixing, the dough is molded into loaves and allowed to rise in warm air, the bubbles of gas in it swelling. It bakes into a bread which in lightness and flavor cannot be distinguished from that made with yeast.

Beer Making.—The fermentation of sugars to form alcohol is another old household art which has passed, in the present generation, into the hands of large factories. The starch in grains (barley) is converted into sugar which is then fermented by the action of the yeast, the percentage of alcohol varying with the organisms used. The maximum amount of alcohol is about 20 per cent while the minimum is about 3 per cent.

Industrial Alcohol.—This is one of the most important chemicals used in manufacturing, absolutely necessary for essences, extracts, and tinctures in pharmacy, and in the paint and varnish factory. The source of much of this alcohol has been cull potatoes from the great potato-growing regions, but corn is also used in the Middle West region where it is the cheapest source of starch.

Other chemicals almost as important can be obtained from the fermentation process, one of them being glycerine. Pasteur[24] noticed that glycerine was one of the substances always found in fermented sugar solutions (3.64 parts) but it remained for later investigators to increase the small per cent which usually is found (about 3 per cent of the sugar fermented) by increasing the alkalinity and by the addition of sodium sulphite.

In order to prevent the growth of the lactic-acid bacteria in these alkaline solutions, sodium or calcium sulphite is added which further helps the production of glycerine so that 20 to 25 per cent of the sugar fermented may be changed into glycerine.

Another valuable chemical which may be obtained in the same fermentation is acetic aldehyde according to the equation

$$C_6H_{12}O_6 = C_3H_8O_3 + C_2H_4O + CO_2$$

The acetic aldehyde unites with the sulphite from which it may be separated. Alcohol and carbon-dioxide production is diminished by the addition of the sulphites.

Fat is produced in quantity by the yeast cells under special conditions, some species producing large amounts. The commercial utilization of this product is, however, still for the future.[14]

YEAST AS FOOD AND MEDICINE

Yeast is today being used as a food and medicine as well as a producer of carbon dioxide in bread making, or of alcohol for drinks or industrial purposes. *S. cerevisiæ* has been found to be very rich in vitamin B, one of the necessary water-soluble vitamins. The proteins in the yeast plants are of types which seem to be very valuable in nutrition, being especially rich in nucleoproteins, contain phosphorus, and go to the building of new nuclear material. Yeast, at the present time, has been given a place in the remedies prescribed by physicians for skin diseases, such as boils, carbuncles, acne, etc., gastrointestinal disturbances and constipation. The recommended dose is about a third of a compressed yeast cake twice a day. Yeast has a tendency to make the bowels looser, especially if taken in a glass of water, intestinal sluggishness being stimulated so that food is forced more normally along the intestine. The yeast cells undergo rapid degeneration and death in the intestines, only a small percentage remaining viable,[25] so that the therapeutic effect is really not known, but it may be partly by the fermentation of excess sugar to form small quantities of alcohol which can be assimilated and used.

As a Food.—Dry as well as compressed yeast serves as a supplementary food, the fact that the organisms are nearly all dead not affecting its value as a food nor its vitamin B.

Dried yeast has been suggested as a supplementary protein ration. Since a yeast cake weighs about ½ ounce (15 grams), a person eating a cake before each meal will add about 6 grams of dry protein to his diet, but as the protein requirements are about 90 grams a day, this amount would be only a small portion of the total needed. Any single form of protein, however, may not supply all of the 18 or more amino acids needed for the proper growth of the body. The claim is made that the yeast protein is complete in that it contains, according to analyses, all of these building stones for the human body; even milk, which will supply all the essentials for growth will not furnish enough fibrin for reproduction.[21] The proteins in the yeast which comes from the great breweries in Germany is dried and used as a very valuable cattle food, while an English medical commission suggested adding yeast to the diet of the British soldiers, 100 grams a day of dry yeast yielding about the same protein as 1 pound of fresh meat. The glycogen which the yeast contains is the same material which occurs in the liver as a storage carbohydrate but is doubtless of minor importance in food value, as compared to the proteins.

The yeasts are a group of plants which are able to form amino acids and proteins from the simplest of chemicals, such as ammonia and sugar. During the World War when proteins and fats were scarce in Germany, the yeast plants of the breweries and distilleries supplemented them with only a small loss of the food value of the carbon compounds from which the yeast derived the energy necessary for the conversion and assimilation. In alcoholic fermentation, the yeast develops the necessary

energy for the synthesis of the proteins which compose yeast protoplasm from inorganic salts. While the cultivated yeasts are unable to make use of an amino acid, such as tyrosin, as a source of nitrogen, they can use nitrate or ammonium compounds.[4]

REPRODUCTION IN THE YEAST PLANTS

1. Budding

The yeast plant usually reproduces by a special form of division called *budding*. The mother cell attains about an average size when a small growth protrudes from one side or end. The material from the mother cell flows into this small bud which increases in size until it may be as large as the mother cell and indistinguishable from it. If undisturbed (Fig. 64), the two cling together for some time and other buds

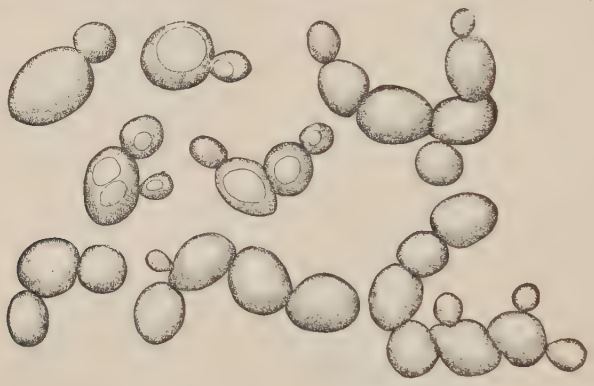

Fig. 64.—*Saccharomyces cerevisiæ* budding in cultures. × 500.

may appear so that an elaborate aggregate of cells is formed; the slightest disturbance in the culture medium, however, shakes the individuals apart and they float away as separate plants. The essentials of this process are not different from those in a mold hypha made up of a row of cells, but in the yeast the attached cells break apart easily; in the former, they remain adhering to form a long hypha. In intermediate types, such as Mycoderma, the cell attachments are not secure and the hyphæ break up readily into individual cells.

2. Endospores

Under certain conditions, the true yeast plants form spores inside the cells (Fig. 65), usually four in number but sometimes eight. Spore formation is preceded by a double division of the cell nucleus to form four nuclei, each of which surrounds itself with a little of the cytoplasm of the old yeast plant. Each spore forms a wall of its own so that the old cell wall now encloses four small spores. The cytological aspects of this nuclear division and spore formation will be discussed later.

In some varieties of yeasts, the formation of endospores is preceded by a fusion of two of the yeast plants, the fusion cell giving rise to the endospores. The group in which this preliminary fusion occurs is known as the *Zygosaccharomyces*. The sexual fusions have been lost in the *Saccharomyces* but the endospores are considered to be of the sexual type.

Conditions for Endospore Formation.—This formation of endospores has been known since the time of Th. Schwann in 1839, but it remained for Hansen[12] (Fig. 66) to work out the physiological conditions under which they were formed. Young,

actively growing cultures of the yeast are placed in an abundance of moisture at a rather high temperature, about 35°C. (about 25 to 30°C. for *S. cerevisiæ*), dependent on the species, with little food but with plenty of air. These conditions are supplied by spreading young cultures on blocks of plaster of Paris in deep Petri dishes,

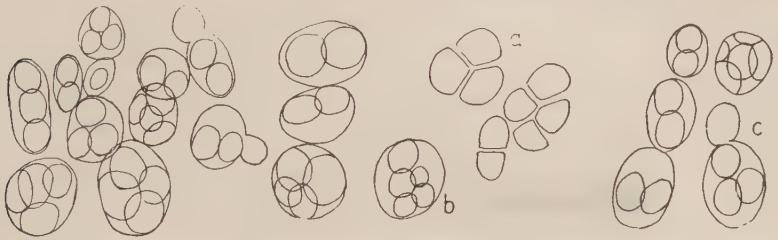

FIG. 65.—Endospores in *Saccharomyces cerevisiæ*: *a*, cells with enclosed endospores; *b*, cell with abnormal (more than normal) number; *c*, cell with rudimentary endospores. × 500. (*After Hansen.*)

the block standing out of the water but with its base immersed about 1 centimeter. Beijerinck[1] used 2 per cent agar; boiled with distilled water; allowed to putrefy; cut up into pieces and allowed to soak in water for 1 week; washed; melted again; and tubes filled on which the yeast was grown. A temperature of 21 to 25°C. was most for favorable for endospores in the ordinary yeasts.

A great change in the hydrogen content is necessary to induce the cells to form spores, the best pH of their formation being about, 7.2 (nearly neutral). Cultures which have been kept in the laboratory for 2½ years show a falling off in the spores (from 75 to 2 per cent) but if the cultures are dried out, the proportion of spores forming cells rises again almost to 100 per cent.[23]

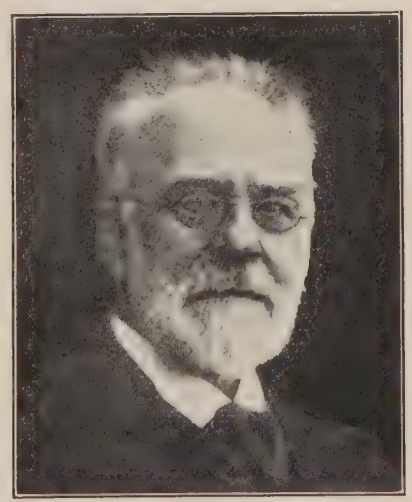

FIG. 66.—Emil Christian Hansen (1842–1910). Discoverer of many important facts in life histories and activities of yeasts.

Beijerinck,[1] observing that yeast cells in cultures gradually lost their power of producing spores, believed that this power of producing spores could be regenerated by growing them on his special agar after selecting cells which still showed the ability to form spores. Ordinary yeast does not seem very favorable for the formation, of endospores but Maneval[19] obtained them easily for class demonstration by simply allowing Fleischmann's yeast cakes to remain for a few days in the ice chest or laboratory. The whitish layer which forms over the cake contains an abundance of cells with spores. On potato-dextrose agar containing 2 per cent of dextrose they appear in 4 to 6 days, while on an agar with the following constituents: 1,000 cubic centimeters distilled water, 3 grams Liebig's extract of beef, 5 grams NaCl, 25 grams dextrose, 20 grams agar, spores develop abundantly in 4 to 5 days and, after 10 to 12 days, over 50 per cent of the cells contain them. Spore formation seems to result from lack of food in the culture medium.

Hansen's Single-cell Method for Obtaining Pure Cultures.—We owe to Hansen[12] the method by which it is possible to obtain with absolute accuracy pure cultures of a yeast. Dilution and plating on agar or gelatin does not insure that the colony obtained may not have originated from two organisms of different varieties which fell near each other in the solid medium. Hansen overcame this difficulty by making such a high dilution that the cells were separated as completely as possible. Some of this dilution in wort gelatin was spread out on a large cover glass and then laid on the vaseline-coated ring of a Bottager slide, a slide with a small ring of glass fastened to it. The suspended gelatin formed a small culture dish. This slide was then placed on the stage of the microscope for examination with the low-power objective for single yeast cells isolated in the gelatin. When such were found, their position was marked on the upper surface of the cover glass with India ink. The slide was then incubated and if one of these marked cells grew into a clump of yeast, it could be removed with a sterile needle into other media as a pure culture originating from a single organism. Lindner[17] improved this method by putting a dilution of a culture in media into a sterile drawing pen with which he drew lines on a sterile cover glass. The individual cells were marked as in Hansen's method and allowed to grow, but isolation was easier from lines than from large drops of culture media.

THE CYTOLOGY OF THE YEAST CELL

In a Living Condition.—The spherical or slightly oval cell is enclosed by a wall composed, according to Brefeld, of fungus cellulose of the same composition as that found in other fungi; cellulose impregnated with chitin. Mangin[20] has claimed the wall was callose but it is so difficult to test chemically that Van Wisselingh,[26] who found chitin in almost all of the fungi and cellulose in many of them, was unable to get a positive test for either of these substances in the yeast-cell walls, and Wettstein[28] could get no chitin test.

The contents of the cell are not homogeneous, as numerous small, highly refractive granules are imbedded in the cytoplasm and one or more vacuoles may usually be found. If only one vacuole is present, it may be a very large one occupying most of the cell space (Fig. 63).

Stained with iodine solution, glycogen, the reserve food, is at first in the form of little granules scattered through the cytoplasm.[27] These gather into a number of vacuoles and finally into one or two large vacuoles. This glycogen is of the same type as that which is found in the animal body and gives similar color reactions, with iodine, red-brown, and not blue, as does starch. The amount present depends on the condition and age of the cultures, disappearing almost entirely at the time of the greatest vegetative activity and then slowly reforming in the cells. These glycogen-filled cells seem to be very vigorous and live longer than any of the others, probably due to their stored food material.

Stained with Methylene Blue.—Stained with an aqueous solution of methylene blue, another set of granules appears, the so-called *metachromatic granules* of Guillermond,[8] the *volutin* granules of Meyer.[22] These granules are protein in nature but are not nuclein according to both Meyer and Wager. They are, however, related to nuclein and may be a combination of nucleic acid with some unknown base. They vary in number and size, depending on the metabolism of the yeast cell, but are most abundant about 40 or 50 hours after fermentation has begun. They disappear as glycogen begins to gather in the large vacuoles. They also vary in number in the different varieties of yeasts, being most abundant in the beer yeasts and in Mycoderma, while few or none are found in the wild apiculate species.[29]

Fixed and Stained to Show the Nucleus.—In yeast fixed and stained to show the nucleus (Fig. 67), the densely colored body which appears at one side of the large

central vacuole is a homogeneous mass, according to Wager and Peniston[27] but Guillermond[8] maintains that it is composed of a nucleole, a network, and chromatic granules. Wager and Peniston claim that the real nucleus is the large central vacuole and that into it extend strands which bear the chromatin granules. Figure 67 shows the two interpretations of the nucleus both at rest and in division to form a new nucleus for the budding cells.

Guillermond finds Wager's chromatin, outside the vacuole, to be only metachromatic or volutin granules. Wager denies that the volutin is in the vacuole; it is nearly all in the cytoplasm although many of the pieces are closely applied to the vacuolar wall. The chromatin granules of his nucleus stain differently and are not metachromatic granules. Wager's nucleus is entirely without a nuclear membrane but such a nucleus occurs in primitive forms. While both agree that the division figure is a very simple one, Wager believes it to be amitotic. One of the two nuclei passes

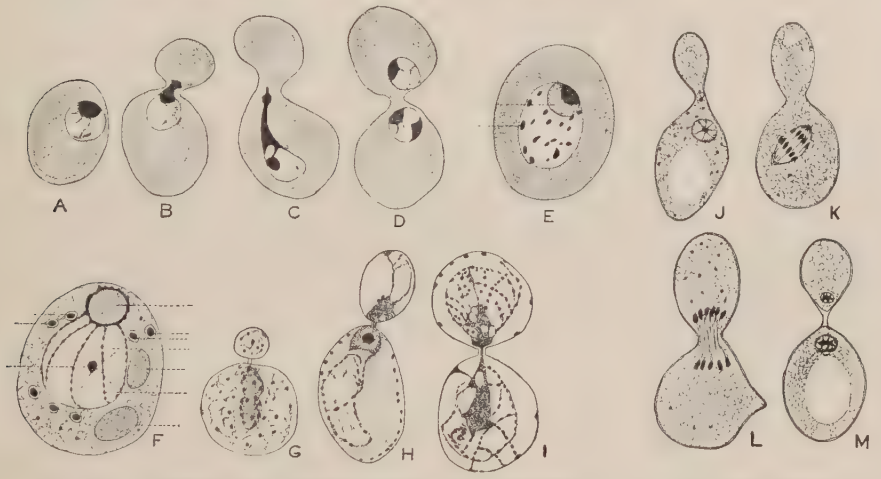

FIG. 67.—Cytology and nuclear division of *S. cerevisiæ*. (*A* to *E*, *after Guillermond; F* to *I*, *after Wager); J* to *M*, mitosis with spindles and chromosomes in yeast (*after Kater*).

out into the growing bud, the other remains in the mother cell. In spore formation, essentially the same process occurs, the nucleus dividing twice to form four nuclei round which the protoplasm collects to form the four spores. It is not possible to say which interpretation is correct, but Guillemond's is analogous with that found in other fungi supposed to be related to yeast, and has had a wider acceptance.

Kater,[16] Fuhrmann,[7] and other investigators find regular mitosis, such as occurs in other plants, with about eight chromosomes. The spindle lies partly in the bud and partly in the mother cell, and thebackward movement of the chromosomes is rather difficult to follow as the spindle is much pinched at its middle (Fig. 67, *J-M*). The resting nucleus contains a nucleole and chromatin, as in the majority of the plants and animals.

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CHAPTER XIV

THE PHYSIOLOGY OF THE YEAST PLANT

The Rate of Growth after Transfer.—*Each strain of yeast has an optimum temperature* at which growth is most rapid. The length of time necessary for a generation of yeast is 60 to 80 minutes at optimum temperature.[50]

Growth is favored by a rather acid medium, a pH of 3 to 6, for the ordinary yeasts,[53] although some of the Mycodermas grow best with a pH of 2 to 2.3. For many of the brewery yeasts, the optimum[11] is about pH 4.8; for the assimilation of dextrose,[14] it is pH 4.5.

Tait and Fletcher[54] stored yeast at the very low temperature of −30 to −80°C., a treatment which rendered the yeast partly liquid. The pH of this yeast juice was 6.2.

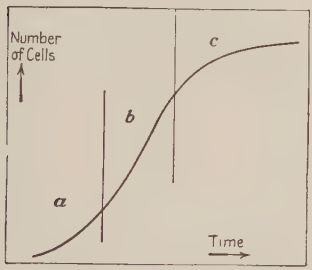


Fig. 68.

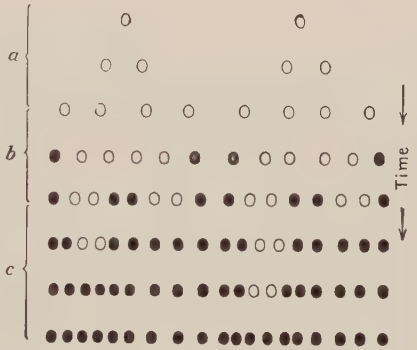


Fig. 69.

Fig. 68.—Growth curve of yeast (diagrammatic). In region *a*, growth is slow and is an exponential function of time; in *b*, rate is directly proportional to time; and in *c*, there is a rapid falling off in the growth rate. The curve is of the S-type. (*After Priestley and Pearsall.*)

Fig. 69.—Explanation of curve in yeast growth. Black cells have gone into a resting condition or are dead, while the clear ones are continuing to grow. As colonies become crowded in *b* and *c*, a majority of the cells go into a resting condition, owing to the increasing abundance of excretion products, alcohol and carbon dioxide. (*After Priestley and Pearsall.*)

The rate of cell increase, after transfer to new culture media, follows definite growth periods, so that the curve has the shape of a letter S. The explanation offered for the S-shaped curve by Priestley and Pearsall[39] is that many of the cells which are actively growing in the beginning, cease growth and go into a resting condition (Figs. 68 and 69).

The increase in the volume of the cells produced during the first 15 hours exceeds the increase in number but, at about 90 hours, the increase in both volume and size is about the same. The individual cells produced during early growth must be larger in volume than the later ones.[41]

The Effect of the By-products on Growth.—Both alcohol and carbon dioxide, the two most important products of sugar fermentation, exert a retarding effect on yeast

growth and finally stop it; carbon dioxide also excludes the air, and oxygen is necessary for a maximum yield.

The Relation of the Yeast Plant to Alcohol.—The various strains of cultivated yeast differ in their toleration of alcohol, some thrive with 5 per cent alcohol in the nutrient medium, others stop with a 10 per cent addition, and a few even will continue until the alcohol content is 12 per cent by volume. Brefeld[7] had to add 17.3 per cent to his culture media in order to prevent fermentation, but it is evident that some of the wine yeasts were present and these withstand more alcohol than the brewery varieties of *S. cerevisiæ*. Temperature affects the amount of alcohol produced by any yeast, the lower the temperature, the larger the amount of alcohol produced. In beer making and wine making, practical use is made of this peculiarity of the yeasts by keeping them in cool cellars where the maximum amount of alcohol can be produced from the materials furnished. Müller-Thurgau[32] with one strain of yeast found that fermentation was stopped:

At 36°C. when the alcohol content was 3.8 per cent by weight.
At 27°C. when the alcohol content was 7.5 per cent by weight.
At 18°C. when the alcohol content was 8.8 per cent by weight.
At 9°C. when the alcohol content was 9.5 per cent by weight.

A yeast can adapt itself somewhat to increased alcohol content but only within rather small limits. The cells which have ceased to grow and divide as the result of a too high alcohol content are not killed but fall to the bottom of the vessel (Müller-Thurgau) and give rise to a new crop of daughter cells which are more resistant. If they are stirred up, so as to get up into a layer of culture fluid with lower content of alcohol than that which hangs over the bottom of the vessel, they are renewed, ferment, and produce more alcohol.

The various alcohols vary in their toxic effect on the yeast plant, the toxicity being dependent on the number of carbon atoms present in the alcohol used.

The per cents (of volume) of various alcohols necessary to check fermentation are shown in the following table based on the incomplete results of Regnard[40] and Yabé[57]:

Carbon atoms	Methyl	Ethyl	Propyl	Butyl	Isobutyl	Amyl	Hexyl	Octyl
	C_1	C_2	C_3	C_4	C_4	C_5	C_6	C_8
Solubility	Any per cent	Any per cent	An per cent	Miscible	Miscible	3 per cent		
Regnard's results	20	15	10	2.5	...	1.0	0.2	0.1
Yabé's results	..	..	..	...	3.0	1.0	...	0.5

The Relation to Carbon Dioxide.—The carbon dioxide stops growth largely by the exclusion of the air. Pasteur[38] believed that the yeast plant would grow entirely without oxygen but as Brefeld[7] was the first to point out, a certain small amount of oxygen seems necessary to induce fermentation to attain its maximum: 1 part of oxygen to every 6,000 parts of carbon dioxide produced. Hansen[15] showed that a beer yeast when grown in beer wort for 60 hours increases 11.2 fold when not aerated; 15.8 fold with an abundance of air. The required oxygen is not very large and a considerable portion of it may be obtained by the yeast plants from the oxygen absorbed in the culture medium but in order to obtain a maximum growth, some oxygen must

be forced through the growing cultures. Harden and Rowland[16] found that for each gram of pressed yeast produced, 3.54 cubic centimeters of oxygen was absorbed per hour and that the amount of oxygen which the culture might absorb could be increased, since the yeast cells would use this supplied oxygen and not depend on the remains of other yeast cells.

The Pasteur theory of fermentation, that a carbohydrate is destroyed by the yeast for the oxygen which it contains, is still partially tenable. A current of air passed through a fermenting sugar solution will change the character of the respiration from anærobic to ærobic, 1 part of sugar protecting 4 to 6 parts from fermentation and fermentation is from 3 to 4 times as rapid under anærobic as compared to ærobic conditions.[29] The yeast plant, while it is usually anærobic, can readily adapt itself to ærobic life and the fermentation of sugars is not necessary for its respiration. The maximum yield of yeast, however, would seem to be produced under the anærobic conditions.

The question seems to be still open, however, as to the value of the oxygen itself as compared to the removal of the carbon dioxide. Carlson[8] increased rapidity of growth about 12 per cent by the introduction of air and about 28 per cent by nitrogen but lowered the rate by using oxygen, 15 per cent, indicating that an abundance of oxygen was detrimental to growth.

The Use of Various Sugars.—Glucose, maltose, and saccharose are all easily fermentable but very few of the yeasts can utilize lactose. Maltose is the best adapted for yeast growth and can be utilized by nearly all yeast species. The pentoses, such as arabinose ($C_5H_{10}O_5$) can be used by some varieties if the culture medium is kept neutral, pH 6.5 to 7.0, instead of the pH 4.5 which is best for the assimilation of the hexose sugars.[1]

Sugar is not absolutely necessary for the process of fermentation as the yeast will grow in the absence of all sugars,[5] but utilizes sugars if any are present in the medium.

The Chemical Changes Induced by the Yeast.—The fundamental equation for the chemical changes which yeast induces in a culture solution containing glucose

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

probably only indicates the more important end products and does not account for a number of other chemicals which appear, at times, in considerable quantities. The reactions can take a number of different courses, depending on the chemicals present in addition to the glucose. Among the more important substances formed are glycerine and succinic acid. The products of fermentation and their calory value are given by Rubner[46] as follows:

From a culture solution containing 100 grams cane sugar with a calory value of 396.8, the following chemicals will appear:

51.1	grams alcohol with a calory value of	358.36
3.4	grams glycerine with a calory value of	14.38
0.65	gram succinic acid with a calory value of	1.99
1.3	grams miscellaneous with a calory value of	5.15
49.2	grams carbon dioxide with a calory value of	0.00
	Calory value of the products	379.88

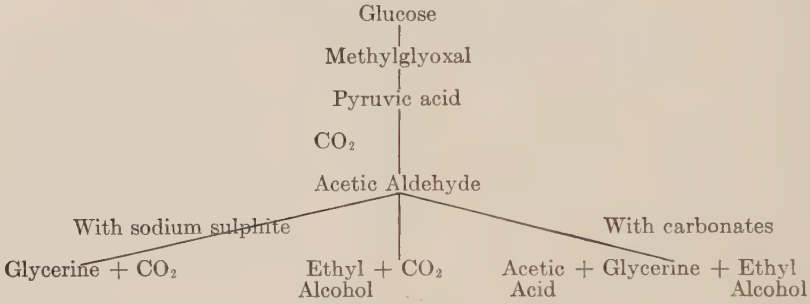
The other 16.92 calories are lost as heat.

The problems involved are chemical and two biological chemists have offered explanations so widely divergent that any attempt at a reconciliation of the two theories seems impossible: the *pyruvic-acid fermentation* theory of Neuberg[33,35,37] and the *sugar-phosphate theory* of Harden.[19] The very complicated Neuberg *pyruvic-acid* theory involves the formation and destruction of pyruvic acid as one of the inter-

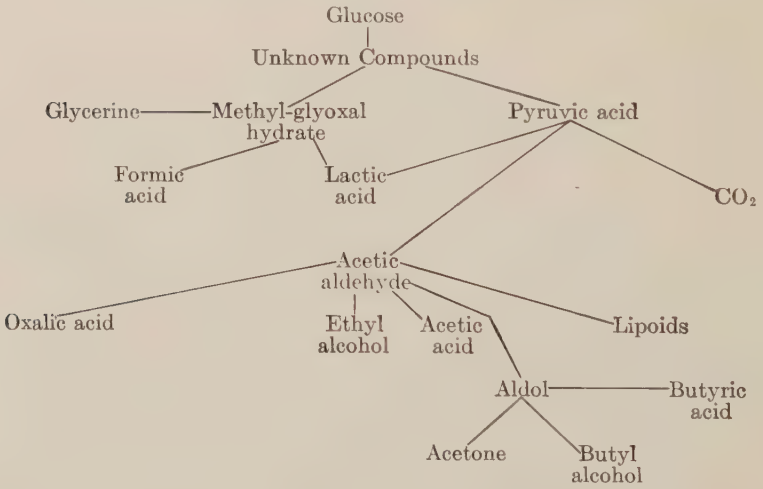
mediate compounds between glucose and ethyl alcohol, while the Harden *sugar-phosphate* theory implies that sugar phosphate is the necessary intermediate chemical formed.[17,20,49]

The Pyruvic-acid Theory.—The pyruvic-acid theory attempts to explain not only the reactions which occur in yeast fermentations but also similar ones due to the activity of bacteria. The reactions are somewhat complex but the following schemes give some idea of the suggested products which arise from glucose fermentation:

YEAST FERMENTATION OF GLUCOSE



Bacterial fermentation of glucose (adapted from Schoen)



Many of the above compounds are known from laboratory experimentation to be formed in the fermentation process but others are hypothetical and their relations are largely theoretical.

The two important compounds are pyruvic acid and acetic aldehyde. Claims have been made that pyruvic acid has been demonstrated as an intermediate product of the fermentation but it is so readily and quickly fermentable by yeasts and bacteria that its "fixation" has been disputed. Acetic aldehyde may be formed from pyruvic acid by decarboxylation with the evolution of carbon dioxide. Acetic aldehyde is very transitory but its presence is claimed to have been demonstrated by fixation with sodium sulphite.

With yeast, fermentation may take one of several courses: (1) glucose may give rise to methylglyoxal hydrate (hypothetical product) which splits into carbon dioxide and pyruvic acid, the pyruvic acid breaking up into ethyl alcohol and carbon dioxide; (2) the glucose may give rise to acetic aldehyde which, in turn, is converted into glycerine and carbon dioxide; (3) the acetic aldehyde may yield acetic acid, glycerine, and alcohol.

If sodium sulphite is added to the culture solution, acetic aldehyde is fixed and accumulates, the more sodium sulphite added, the more acetic aldehyde fixed. The acetic aldehyde gives rise to glycerine and carbon dioxide. The trend of the reactions can be turned to the production of glycerine, therefore, by the addition of a high percentage of sodium sulphite. With carbonates added, the acetic aldehyde forms acetic acid, glycerine, and alcohol.

Pyruvic acid may not occur at all in normal fermentation in which ethyl alcohol is the principal product; methylglyoxal hydrate may give rise directly to ethyl alcohol without its formation.

Much attention is being given to the fermentations suggested by these schemes, not only by the yeasts but also by the bacteria. The bacteria, in some instances, form other organic compounds and their occurrence must be explained in some manner, such as the scheme suggested by Schoen.[49]

The Sugar-phosphate Theory.—According to this theory, the reactions take place in two phases: in the *first*, one of the phosphates takes a part to unite with the hexose, and in the *second*, this hexosephosphate sugar breaks down into alcohol and carbon dioxide through the action of *zymase*. The equations representing the process are as follows:

$$2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_6O + 2H_2O + C_6H_{10}O_4(PO_4R_2)_2 \quad (1)$$

$$C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4 \quad (2)$$

R may be any soluble salt of phosphoric acid, such as sodium or potassium phosphate. The hexosediphosphoric acid formed in the first equation is hydrolyzed by the agency of an enzyme to form again glucose and phosphate, the sugar being of a fermentable type. The addition of phosphates to a culture solution hastens the reaction, since the first equation must occur before the yeast can ferment the sugar. The phosphate acts as a catalytic agent but is not the coenzyme necessary for the working of the enzyme, zymase.

The addition of a phosphate to the mixture of macerated yeast cells, without living cells, increases CO_2 production with a hexose phosphate formed. After this phosphate is used up entirely, the addition of more phosphate will again start the reaction. The addition of phosphates to bread dough is not always necessary, since the wheat grains are especially rich in the phosphates, but as much of this phosphate containing material is removed in modern systems of milling, it has been found that the addition of some of the inorganic phosphates will hasten yeast action and produce lighter bread.[4]

The Harden theory is not nearly so complicated as the pyruvic-acid theory but Neuberg[34] is very doubtful as to the importance of the hexosediphosphate formation; he admits that it is marked with yeast juice but not nearly so effective with the yeast cells. It may be a pathological process but the whole fermentation of sugars is put by some investigators in this class of phenomena.

The Enzymes in the Yeast Plant.—The marked chemical changes which the yeast plant induces in the medium in which it is growing are carried out by the activity of its enzymes. The enzymes transform the food in the substratum into a form which can be utilized in yeast-cell growth; life without enzymes would be impossible.

The cells of molds, yeasts, and bacteria are full of enzymes which serve the protoplasm which produces them in various ways, and make possible that complex of

processes which we call "life." Still, we must not regard the protoplasm merely as a complex of enzymes (Palladin). "Enzymes are, in a manner of speaking, workers in the service of the protoplasm; they are formed by the protoplasm, used in the work that is in hand, and then imprisoned or destroyed as soon as their activities are no longer required. Enzymes that have become unnecessary are rendered inactive by specific anti-enzymes; they are imprisoned, as it were, and when once more they become necessary, they are rendered active again, from their condition of proenzymes, by activators or kinases. Activators or kinases, on the one hand and anti-enzymes on the other are thus agents through which the regulatory power of the protoplasm is effected." Enzymes sometimes act only effectively in association with other parts of the cell or at least attached to bits of protoplasm.[52]

The yeast plant is one of the most abundant and interesting sources of enzymes and many of the facts known about them have been derived from the study of the types which it forms.

The four types of enzymes abundant in yeast plants (especially the cultivated species) are *zymase, sucrase, maltase,* and the *proteolytic enzymes* which act on the gluten of the flour. The first of these, *zymase,* is the agent which brings about the destruction of glucose to form alcohol, according to the equation

$$C_6H_{12}O_6 + \text{zymase} = 2C_2H_5OH + 2CO_2 + \text{zymase}$$

The sugar which can be used in this equation must be one of the hexose sugars, but the disaccharoses must be first simplified. The yeast is provided with an enzyme for the conversion of these sugars into an invert sugar according to the equations

$$\underset{\text{sucrose}}{C_{12}H_{22}O_{11}H_2O} + \text{sucrase} = \underset{\text{dextrose}}{C_6H_{12}O_6} + \underset{\text{fructose}}{C_6H_{12}O_6} + \text{sucrase}$$

$$\underset{\text{maltose}}{C_{12}H_{22}O_{11}H_2O} + \text{maltase} = 2\underset{\text{dextrose}}{C_6H_{12}O_6} + \text{maltase}$$

The process in both of these equations is a hydrolytic one. The equations which are given are probably not entirely correct, as products are formed of which no account is given. Other sugars may be utilized and the yeast enabled to use each of them by a specific enzyme.

The proteolytic enzymes[5] of the yeast plant are available for the splitting and using of the glutens of the flour. They are of less importance than the sugar-splitting types but are of value in producing soluble proteins.

The Bios Theory.—Wildiers discovered[59] that one of the beer yeasts of the *Saccharomyces cerevisiæ* type, inoculated into a culture solution containing sugar and the necessary mineral salts (with the nitrogen in the form of ammonium chloride) and the inoculum very small in quantity, would grow, but very poorly. But, on the other hand, rapid growth could be secured if the inoculum were two drops of a beer-wort-transfer into beer wort or if 0.25 to 1.0 cubic centimeter of pressed yeast broken up in water were substituted. Abundant growth could also be induced by the addition of a few cubic centimeters of boiled yeasts, Liebig's meat extract, peptone, or beer wort. These results were so striking that Wildiers assumed that there must be some substance supplied by the yeast inoculum, the peptone, or the meat extract which was absolutely essential to the growth of the yeast cells and which was not found in inorganic salts or in the sugar which composed the synthetic medium.

This hypothetical substance does not occur in the ash of the yeast plant; can be destroyed by boiling with 20 per cent sulphuric acid so that it will not function; is dialyzable; is water soluble; and can be extracted from the yeast cells by boiling. Since it came from living organisms it was designated by Wildiers, *bios.*

Such a radical statement at variance with all the dogmas of yeast nutrition, propounded by Pasteur, laid Wildiers' work open to sharp criticism. Various explanations were offered as to the cause of such peculiar results: that the distilled water

which Wildiers used contained copper from the copper coils of the stills or that there were traces of ultramarine in the cane sugar used, since this chemical is sometimes found in the cane sugar of commerce. He had, however, also secured similar results with glucose in which this chemical does not occur. The nutrient solution seemed to contain a poison to the yeast when the yeast was present in small numbers in the new environment, but Amand[2] showed that the bios did not act as an antipoison.

While Wildiers' experiments were somewhat quantitative, neither the inoculums nor the yields from them were tabulated, although he did record the amount of carbon dioxide produced under the different conditions. Kossowicz,[22] using a single *S. ellipsoideus* yeast cell for the inoculum, found that in 21 trials out of 22 the cell died, while in the single instance where growth occurred, an unusually large amount of the wort gelatin had been accidentally carried over. With 200 yeast cells used to inoculate the culture solution (100 cubic centimeters of sugar-mineral salt composition) the numbers would increase in 50 days to 140,000,000 cells.

Wildiers' inoculum, since he used commercial yeasts, had been obtained from a brewery and might have contained *Mycoderma vini*. Winogradsky[62] had already shown that this Mycoderma could live on pure ammonium salts as a source of nitrogen. With small inoculums the chances were very remote that any of this Mycoderma would be included; with large ones, some of it might be added and start the growth of the impure culture.

The Bios Theory and Pasteur.—The bios controversy has thrown some interesting light on an old dispute between Pasteur and Liebig. Liebig's theory of fermentation (based on the lability of the protein and carbohydrate molecules) required a vigorous attack, such as could be supplied only by a number of yeast cells; one or two cells would have no effect. Pasteur's theory, however, was based on the assumption that only one cell was necessary for all of the changes in pure inorganic solutions and there was no place nor necessity for food of organic origin.

Pasteur claimed that the ashes of yeast would furnish all the necessary nutrient salts for abundant yeast growth and that all that was necessary to make a complete food for successful growth was to combine them with ammonium salts and sugar as sources of nitrogen and carbon; but when Liebig,[24,25] using the same formula but inoculating with a minimum of yeast, was unable to secure similar results, Pasteur offered to produce any quantity, "all the yeast he could require," using a small inoculum and his yeast medium. Liebig declined to make the trial and Pasteur was considered to have won his point.

The Bios Theory and Vitamins.—The bios theory drifted along with scattering experimental work upon it until the growth stimulating and diet accessory substances, *vitamins*, were discovered, when a fresh burst of experimentation started. Bios and vitamins were both apparently necessary for growth, so the assumption was made, on the basis of some experimental evidence, that the two were very similar if not the same; the fact that yeast contains an abundance of vitamins probably also helped out the suggestion.

The literature has become so extensive[55] that only the results of a few investigations can be noted to show how the problem has presented itself to later workers.

Experimental data have been gathered to answer a series of questions: (1) Will growth occur at all if a very small inoculation is made in pure inorganic media plus some of the simpler organic compounds, such as sugar? (2) Will some compounds of living origin stimulate growth when only a few organisms are added? (3) What must be the nature of this added supplementary material; does it have to be one of the vitamins?

Williams[60] was unable to grow *S. cerevisiæ* successfully when inoculated in very small quantities into a synthetic sugar solution, although it grew abundantly with

the addition of various organic compounds containing vitamins. He devised the yeast method as a test for antiberiberi vitamins.

Robertson and Davis[45] inoculated single yeast cells into a solution in which asparagin and dextrose were the source of nitrogen and carbon without securing growth, but the same inoculation did well if a little of the water-soluble material from a beef heart, from carrots, potatoes, or other yeast cells were added. Without this addition, the amount of growth is only proportional to the number of cells inoculated. Lepeschkin[23] has shown that a single-celled culture of yeast made in media in which ammonium sulphate was the source of nitrogen would grow abundantly if only 0.001 per cent of crystallized enzyme, vitamin B (antineuritic), obtained from yeast by Funk's method were put into the culture. In hanging-drop cultures inoculated with 2 to 3 cells, a distinct increase in growth rate could be seen if 0.0001 per cent of vitamin B were added and hyphæ of the green Penicillium, lengthening 200 per cent without vitamin, grew 3,483 per cent if 0.1 per cent vitamin was present. Eddy, Kerr, and Williams[10] isolated from autolyzed yeast a crystalline substance, melting at 223°C., which increased the volume growth of yeast twenty-fold, as compared to controls. This crystalline substance, which they named crystalline *bios 223*, loses its ability to act as a stimulating substance if changed chemically, but regains it if the original chemical condition of the crystals is restored. It is not the antineuritic vitamin.

On the other hand, some investigators have been able to get yeast to grow in the absence of growth-promoting substance. MacDonald and McCollum[28] believed that the yeast plant was able to synthesize its own vitamins to meet its own needs from inorganic media[22] and Fulmer, Nelson, and Sherwood[12] did not find vitamin B necessary for growth.

Miss Bachmann[3] found considerable variation in the vitamin requirements of various yeasts, some needing a large amount for their proper development, others could get along on very little. She agrees, however, that the water-soluble vitamin B will stimulate growth if any stimulation is required.*

The difference in the bios requirements of various strains and species of yeasts receives strong support from Tanner, Devereux, and Higgins[56] and Devereux and Tanner[9], who, instead of confining their attention to the ordinary domestic yeast, have tested out 50 pure species of yeasts and 22 strains of yeast-like fungi with regard to accessory food substances. In a second experiment, 46 varieties were used in purified sugar and inorganic salts. Transfers were made every 7 days for 28 days to free the cells of growth products. Of these varieties, 22 grew well, 2 moderately, 2 scantily, and 20 not at all, with small inoculations and no accessory food substances. Among the yeasts which refused to grow was *S. cerevisiæ*, confirming Wildiers' contention that this species would not thrive in pure inorganic salts plus sugar.

The relation of bios to vitamins is not clear nor is it to the growth-promoting substances of higher plants known as *auximones* or *hormones*. The physiological effects of the latter substances are somewhat the same, as the following work will show.

The higher plants flourish much better in water cultures which contain some organic material in solution, so that the bacteria of the soil supply not only the necessary nitrates of the soil solution but also some unknown organic material which seems to stimulate their growth. Miss Mockeridge,[31] from experiments with the remains of yeast and bacteria (Azotobacter), has come to the conclusion that these organisms supply some substance which is a real stimulant and not a food, its effect being out of all proportion to the amount supplied. The nature of this stimulating substance is unknown, but the materials added contain all of the necessary radicals for the formation of nucleic acid.

The presence of some of these organic compounds seems to be absolutely necessary for the cell growth and division in higher plant tissues. Robbins[42] tried to grow

*See Smith[51] with reference to the naming of this vitamin B.

amputated corn root tips in inorganic culture media with sugar, but they refused to proliferate cells unless some protein, such as that obtained from yeast or peptone, were present; a carbohydrate would not supply this need.

Similar growth stimulants have been claimed in some of the animals such as Robertson[43,44] described in the reproduction of one of the infusoria, (*Enchelys farcinem* Ehr). While attempts to repeat these effects have been unsuccessful, subsequent investigators did not have the same species, and variations may occur among the protozoa as in the yeasts.

Bios and Molds.—Similar stimulation effects have been obtained by some investigators by the addition of vitamins, or at least some substance of organic origin, to mold-culture media, while others claim such accessory substances are useless.

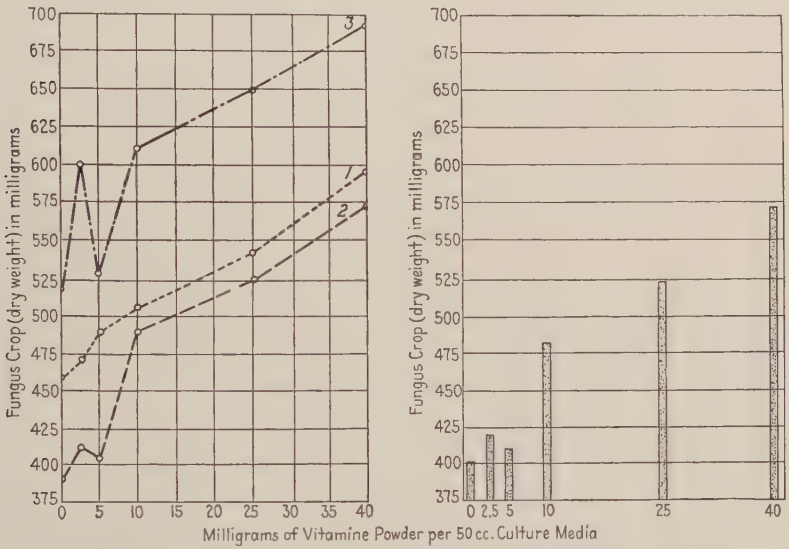


FIG. 70.—Effect on yield of *A. niger* of adding varying amounts of yeast vitamin to liquid culture media: *A*, curve 1, yield after 40 hours; curve 2, after 3½ days; curve 3, after 12 days; *B*, total yield with varying amounts of vitamin. (*After Schelling.*)

Willaman[61] grew the brown-rot fungus, *Sclerotinia cinerea*, in a mineral solution with asparagin used as a source of nitrogen, but found that the yield of mycelium is much more if a little prune juice is added. The prune juice seems to add two vitamins to the fungus. One accelerates vegetative growth, the other spore production; both must be present in order to have complete normal growth and reproduction.

Linoissier,[26,27] on the other hand, as the result of experiments with *Oidium lactis*, *Aspergillus niger*, and a green *Penicillium*, is convinced that the vitamins play no part in the nutrition of these fungi, providing there is an abundance of inorganic minerals in the nutrient medium. An abundance of ammonium salts and glucose will cause the organisms to grow just as well as if they were also supplied with vitamins, such as those in orange juice. If, however, the nutrient solutions are poor in mineral salts, the addition of a little orange juice will, to some extent, help growth.

Miss Schelling[48] found that *A. niger* gave increased growth if a vitamin were added to the inorganic chemicals, the effect being especially noticeable during the first 5 hours after its application (Fig. 70). The stimulation by the vitamin seems to be exhausted after a few days, however, and the dry weights of the mycelium produced after 40 hours are just about the same as those to which the vitamin B had

been added. The stimulation occurs during the first few hours and after that time stimulated cultures and unstimulated ones grew at the same rate.

The same conclusions on molds will probably be finally reached as have been obtained on the yeasts: some species require growth stimulating additions; with others, the yield is just as much without them.

Bios and Bacteria.—The effect of adding bios to cultures of bacteria has been studied on a number of organisms with results as diverse as those described for yeasts and molds, as will be noted later.

The possibility always exists that the vitamin, if supplied in quantity or in a concentrated form, may serve as a supplementary food supplying a deficiency of nitrogen, as Werkman[58] found on cultures of *Azotobacter crococcum* and *B. radicicola*. The dividing line between a *food* and a *stimulant* is not always clear.

General Conclusion as to Bios.—The effect of the addition of various accessory substances seems to vary and considerable uncertainty seems to remain as to the nature of bios. The fact that a single organism, when transferred to a new inorganic medium, frequently does not grow and divide as rapidly as when a number are carried over still remains unexplained.

We may assume that, in a colony, the organisms live in a symbiotic condition, one making use of the by-products of the other, possibly one organism secretes enzymes which may be lacking in the other. Of a number of individuals present, one may perform certain functions better than any of the others, so that it is an advantage to have a community life where not all the responsibility is thrown on a single individual. If the more easily assimilable organic bios is supplied to this lone individual, however, it is able to grow and reproduce rapidly even if some of its assimilatory functions are weak or lacking.

The relations of bios, vitamins, and growth factors (auximones or hormones) is not clear. The physiological results of the addition to culture media of bios and vitamins seems to be somewhat similar, but they do not seem to be identical. While bios may not be as fundamental as it was once thought to be, the work that has been done upon it has added much valuable information on the nutrition, organic and inorganic, of lower organisms.

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CHAPTER XV

THE ENZYMES OF THE YEASTS

Some of the enzymes pass out of the cell into the culture medium and are called "extracellular"; others are retained in the cell and do their work there without appearing in the culture medium, "intracellular." Diastase, already discussed in connection with the molds, is an example of an easily obtained and abundant extracellular enzyme which has been known since 1833.

ZYMASE

The intracellular enzymes, however, are difficult to free from the cells and were not known until Buchner[11] made the announcement that he had succeeded in obtaining enzymes from yeast cells which would ferment sugar without the intervention of the living cells of the plant itself. This discovery was in opposition to the theories of Pasteur[37] that the living yeast cell was necessary to carry out this fermentation and that it could occur only in the absence of all air since the yeast plants obtain their oxygen by the destruction of the sugar in the solution.

Buchner's method was so original that it is worth giving in detail:

1,000 grams of purified pressed yeast was mixed with an equal weight of quartz sand and 250 grams of diatomaceous earth and the whole mass rubbed together until it was soft and plastic. This paste was then well mixed with 100 grams of water and placed in a filter cloth and subjected to 400 to 500 atmospheres pressure. About 300 cubic centimeters of extract was obtained. The pressure cake was again mixed with 100 cubic centimeters of water and 150 cubic centimeters more of extract obtained. About 500 cubic centimeters of extract were obtained from 1,000 grams of yeast which contained about 300 cubic centimeters of cell contents. In order to free it from turbidity it was mixed with 4 grams of diatomaceous earth and filtered through paper. A clear, opalescent liquid, smelling of yeast, resulted. Boiling coagulated it into a solid. It contained 10 per cent of dry matter and 1.15 per cent of ash.

With the yeast extract solution, Buchner was able to furnish many details of the action of this enzyme which occurs inside the yeast cells and which had been deemed to be a property of protoplasm and inseparable from it. This solution would cause fermentation in sugar solutions, so Buchner entitled his paper, "Alcoholic Fermentation without Yeast Cells." The fermentation would still continue if the solution were saturated with chloroform to stop all organized protoplasmic activity, and filtration through a Berkfeld filter did not destroy its power. If a parchment paper dialyzer full of the yeast extract were hung in a 37 per cent cane sugar solution, after a few hours the surface of the paper was covered with CO_2 bubbles and there was an evolution of gas inside, due to the percolation of sugar through the membrane. The activity of the enzyme was short lived, however, as it disappeared in 5 days in the ice box, and in a sugar solution in two weeks. Heated to 40 to 50°C., it lost its power of fermentation. If precipitated by strong alcohol and dried thoroughly over sulphuric acid, it lost its activity. For the enzyme, Buchner proposed the name *zymase*.

Buchner soon found that he did not have a single enzyme but a series of enzymes, among which could be identified sucrase, maltase, and a glycogen-fermenting enzyme.

The sucrase would change cane sugar to dextrose and the maltase would, in a similar way, invert malt sugar.

The zymase was the principal constituent and the one upon which he worked and was most interested. The extract could be passed through a Chamberland filter and still retain its power. He claimed that the loss in fermenting power after storage was induced by peptonizing enzymes which attacked the zymase itself and destroyed it. If the yeast extract were carefully evaporated to dryness at a temperature of 35°C., it would retain its power for some months. It would ferment up to a 50 per cent solution of cane sugar, dextrose, or maltose, but its power diminishes during the first 24 hours and disappears entirely after 3 or 4 days; its action was more rapid at higher temperatures but the enzyme itself was much more quickly exhausted. The action never approached the strength of living yeast cells. Nord and Franke[34] prepared by physical methods a zymase solution which they claim will remain permanently active.

The products of the fermentation were obtained by fermenting 25 grams of cane sugar with 100 cubic centimeters of yeast extract and 2 per cent of arsenious acid at 15°C., later raising the temperature to 50°C. After 24 hours the sugar was all fermented and 12.2 grams of carbon dioxide and 12.4 grams of alcohol produced. Since Pasteur believed that glycerine and succinic acid were constant products of fermentation, the percentage produced by the extract was determined. Müller-Thurgau had maintained that these by-products of the fermentation were not constant in that they arise not from the sugar but from the yeast cells themselves as an excretory waste; a viewpoint sustained by Wortman, who found that whether much or little alcohol was produced, the amount of glycerine was the same. By the use of the extract, Buchner and Rapp[8] were able to get from 100 grams of cane sugar, 50.4 grams of alcohol, 0.5 gram of glycerine and 0.3 gram of succinic acid. The percentage of glycerine and succinic acid was smaller than with the use of the living yeast cells.

With living yeast cells in a solution containing both dextrose and levulose, dextrose is used first and levulose ferments only after the most of the dextrose has disappeared. The explanation offered is that the dextrose is able to penetrate the cell walls more easily than the levulose and is destroyed by the zymase inside the cells. The zymase extract in a solution containing equal parts of the dextrose and levulose used the two sugars equally rapidly since no cell wall had to be passed.

Buchner's theory that zymase is digested by another enzyme of a protein-dissolving nature is correct, in all probability, since the yeast extract, if carefully evaporated until in a concentrated solution, will keep much longer than while in a very aqueous form.[9]

500 cubic centimeters of the crude yeast extract dried in a vacuum at 35°C. will yield a powder resembling egg albumin and weighing 70 grams. This powder is soluble in water and retains its activity. Using this method as a basis, Buchner patented a process for gradually drying yeast at low temperatures, and then at high temperatures, so as not to injure the enzymes contained in it. This product may be ground and added to flour, 5 to 10 per cent being sufficient to raise bread without living yeast cells.

Criticisms of Buchner's Experiments.—The Buchner observations did not escape criticism from other chemists and students of fermentology. Abeles[1] suggested the possibility (1) that bits of the yeast cells were still present in the extract, (2) that the poisons which Buchner considered to be sufficient to be fatal to protoplasm might not work if large quantities of the yeast were added, and (3) that the heating of the yeast extract to 100°C. was no guarantee that all the yeast cells had been killed, as the young yeast cells are very resistant to heat and some of them might have escaped alive.

Buchner was able, however, to obtain the same enzyme out of dead yeast cells, providing the drying was done carefully. Yeast, for instance, which had been heated to 102°C. for 4 hours, then to 110°C. for 5 hours, and, finally, at 120°C. for 10 hours would still yield the active extract, although it was only one-fourth to one-half the strength of that obtained from fresh yeast. It is hardly necessary to say that there was no fermentation from the yeasts treated in these temperatures.

Much of the fermentation induced by zymase solutions, however, may be due to the enzyme clinging to bits of the yeast protoplasm.[42] The amount of zymase is very small in yeast cells that are actively fermenting sugar solutions; it seems to be all used up as it is formed but if the yeast is kept for 2 to 3½ hours, the amount of zymase increases, as it is stored in the cells.

The Activation of an Enzyme by Other Substances.—One of the most interesting discoveries made on zymase since its original discovery by Buchner is that of Harden and Young[23] of a *coenzyme*. When they passed yeast juice through a gelatin filter, so that the colloids were left behind inside the filter, they were surprised to find that neither the substance inside the filter, nor the filtrate, would ferment the sugar solution when used alone, but, if the two were united, the typical reaction would follow. The coenzyme is dialyzable while the enzyme remains behind in the filtrate. This coenzyme is not a phosphate according to these authors but it may be an ester of phosphoric acid.[8] Both phosphate and the coenzyme seem to be necessary for the activity of the enzyme itself.

More Recent Work.—Since Buchner's extensive work on zymase, not so much has been done with the enzyme itself and doubts have been thrown on its importance, Rubner[40] claiming that only from 2 to 5 per cent of the work done in fermentation was by the zymase alone. The enzyme must act in association with the living protoplasm of the yeast or it is much weakened. Its activity alone is very small; in fact, much of the activity that has been observed may be due to the attachment of the enzyme to bits of yeast protoplasm.[42]

The extensive chemical investigations on the fermentation process has further led to the suggestion that zymase is not a single but a complex enzyme. Carboxylase, an enzyme which splits pyruvic acid, according to the Neuberg theory of fermentation, (page 136) is less sensitive to poisons and high temperatures than zymase, being one of these components.[32,33]

The Loss of the Ability to Produce Enzymes.—The yeast plants may lose their power to ferment sugar after they have been kept growing for some time in complete nutrient solutions where an abundance of nitrogen is provided. Albert[2] regenerated this fermentive power in a yeast, following an old suggestion of Hayduck that the yeast plant lost its ability to ferment sugar because it had been fed too much nitrogen, by putting it in a 16 to 22 per cent solution of sugar alone. The stored-up nitrogen in the yeast cells is used as the fermentation proceeds, about 30 per cent of it being lost. The yeast cells then store glycogen in its stead but this substance also disappears after fermentation is completed.

The Autofermentation of the Yeast Itself.—Pasteur made the interesting discovery that the yeast plant could digest itself and produce alcohol and carbon dioxide. 0.424 gram of sugar and 10 grams of yeast will produce, in a solution, not 110 cubic centimeters of carbon dioxide as expected by the equation, but over 300 cubic centimeters.

This additional 200 cubic centimeters of carbon dioxide is not produced, as Pasteur believed, from the cellulose walls of the yeast plants but from the glycogen stored in the yeast protoplasm. In fact, it is possible to produce alcohol and carbon dioxide without any sugar at all in the solution, the glycogen inside the yeast plants furnishing sufficient fermentable material. The sugar resulting from the conversion of glycogen is of the dextrorotatory type.[13] Yeasts without glycogen, such as *S. pombe*, never show autodigestion.[5]

The digestion of the glycogen in the yeast plants by the action of internal enzymes is checked by narcotics, such as chloroform and ethylacetate, while others, such as toluol, hasten their activity.[17] If yeast cells, filled with glycogen and treated with toluol, are placed in a solution of sugar and phosphate, the yield of carbon dioxide is less in quantity and slower to form than from the same amount of yeast placed in distilled water. The sugar in the solution is used instead of the glycogen inside the yeast cells and exerts an inhibitory effect on self-digestion.

SUCRASE

Although the zymase has been somewhat neglected in recent years, the same is not true of *sucrase*, known also as *invertase* and *saccharase*, which transforms sucrose into the invert sugars. This enzyme is produced by the yeast in such abundance that it has been a favorite one for experimentation, and from it much has been learned about enzyme activity.

One of the chief difficulties in all studies of enzymes is to get them in any state of purity; they are precipitated with a mass of other proteins from which it is almost impossible to free them.

Separation and Purification by Adsorption.—Colloidal substances may be removed from solutions by the process of adsorption, on finely divided insoluble substances. Michaelis and Ehrenreich[28] found that kaolin, a very fine-grained diatomaceous earth, would adsorb basic colloids from a suspension or colloidal solution, and that aluminium hydroxide would do the same to acid colloids. Willstätter has been very successful in purifying enzymes in this manner, one of them being sucrase from yeast. The enzyme in solution in water, alcohol (50 per cent), or acetone, is treated with kaolin and aluminum hydroxide. Treatment with aluminium hydroxide alone gave an enzyme one hundred times as strong as that in the original material. The easiest method found of obtaining sucrase in a fair state of purity is to remove the proteins with $N/20$ acetic acid, (diluted twenty- to forty-fold) and adsorb with kaolin. The sucrase is then freed by very dilute ammonia from the kaolin.[47–49]

Euler and Josephson,[18] working on sucrase which they had purified in part by the Willstätter methods, found that they could get the enzyme to a point where it could not be increased in strength. These purest preparations of sucrase were found to be protein in nature and they were able to isolate from it some of the common amino acids found in other plants and animal proteins. Willstätter, Gräser, and Kühn[45] had claimed to be able to purify sucrase until it contained no protein, carbohydrate, or phosphorus. Euler and Josephson[18] estimated the molecular weight of the sucrase to be about 20,000, this weight being based on its rate of diffusion and not obtained by the usual methods.

Purification of Enzymes by Dialysis.—The substances associated with enzymes are usually easily dialyzed, so that they can be separated from the pure enzyme by putting the impure enzyme solution in a dialyzing membrane, surrounded by water, the impurities passing through the membrane, leaving the purer enzyme inside. This method of separation by membranes has been further improved in the passage of electric currents through the solutions. The current carries the enzymes

(each particle laden with a plus or minus charge) to the pole having the opposite charge.[22]

THE CHEMICAL NATURE OF ENZYMES

Although agreement is general that enzymes are colloids, all unanimity of opinion, as to their chemical nature, seems to end here. Willstätter was unable to free his purest sucrase from a carbohydrate which was afterwards shown to be mannose, by Koelle,[26] a view upheld also by Euler, Lindberg, and Melander.[15] The latter found that, while the enzyme contained mannose, it had so very little nitrogen that it could not be a protein. A number of investigators are convinced that the enzyme is really a dual body composed of a colloidal base to which is united a group of atoms which are purely chemical in their action. Both chemical and colloid are necessary for the reaction of the particular enzyme. This composite theory, apparently originally stated by Bertrand,[6] has met favor with many of the later investigators.[27,3,46] This theory implies, too, that the chemical nature of enzymes might be widely different, since the two groups composing them would not necessarily have to be always of the same composition. It would seem likely, too, that one or the other of the two components would be closely related to the substratum on which the action occurred, that is to say, an enzyme acting on carbohydrates might have a carbohydrate constituent; a proteoclastic one might have some form of protein in its composition. The difficulty of obtaining pure enzymes free from other proteins has made, as yet, the problem of determination of the constituents an indefinite one.

The fact that the enzymes are specific in their action, that is, an enzyme will cause only chemical changes in one particular type of chemical substance, led Fischer[20] to suggest his famous lock and key simile to explain or to give a mental picture of what may occur in a reaction where an enzyme plays a rôle. He suggests, in this comparison, that each molecule has a particular configuration and that the enzyme must have a corresponding one if at all possible for it to unite with the molecule in chemical association. They must fit together, as a lock and key.

The Effect of Temperature on the Rate of Action.—Chemical reactions are generally speeded up by an increase in temperature, so that, following the rule of van't Hoff, an increase in temperature of 10 degrees will double the rate of action. The enzyme itself (separate from its colloidal substratum) being purely chemical in its action, behaves as do other chemicals and, in general, follows this rule up to certain temperatures when further increase in the heat applied results in a slowing down of the rate or reaction. Sucrase, like most of the enzymes which have been studied, seems to attain its maximum reaction at about 40°C. Euler and Beth af Ugglas[14] have shown that in sucrase the rate of activity drops off above 50°C. and that at 63°C. the rate of action is diminished 50 per cent, provided the temperature is increased only 0.02 of 1°. The rate shows a gradual decline up to this point which may be considered as the thermal death point.[16]

The death temperature for enzymes varies between 60 and 100°C.; the reaction ceases entirely, due to the destruction of the enzyme itself. If the enzyme is composed of a chemical substance acting on a colloidal substratum, such a destruction of the enzyme is probably the consequence of the coagulation of the colloidal portion.

The Concentration of the Enzyme and Its Rate of Action.—Willstätter, Gräser, and Kühn[45] and Michaelis[29] have found that the rate at which sucrase acts on a sugar solution is directly dependent on the strength of the enzyme; in a given solution of sugar, increasing the amounts of enzyme gives a straight curve. The solution must be a real one, however, and not too concentrated. If the substratum is colloidal, the curve is not simple, or if the concentration of the sugar solutions is so strong that it is a syrup, the reaction is slowed down in the higher percentage, owing to the viscosity of the medium[12] or to lack of water for the reaction.[25] The time element

enters into the reaction since, with a syrup, the particles of sugar must require some time to find their way to the enzyme and, on the other hand, the product must diffuse away from its vicinity so that the reacting surface of the enzyme may be free. Up to the stage that this viscosity factor enters into the reactions, however, the increase in the concentration of the medium means an increase in the rate of the reaction.

Effect of Acidity and Alkalinity on Rate of Action.—Every enzyme has a definite acidity range within which it is active. This range is from extreme acidity for pepsin, to marked alkalinity for trypsin from the pancreas. The action of sucrase is most vigorous[19] at pH 3.7 to 5.2, or at 25 to 35°C. with a pH of 4.5 to 5.[31]

Effect of the Reaction Products on the Rate.—The products of the reaction retard the reaction of an enzyme; thus, Michaelis and Menten[30] found that the addition of fructose hindered the action of the sucrase more than did the same quantity of glucose. Some forms of the glucose hinder the action more than others, although all have the same empirical formula. Armstrong[3] found that, in the case of sucrase, the presence of fructose would slow up its action while this latter sugar would have very little effect on other enzymes. Glucose, however, seems to have very little effect on the rate of change produced by any of the enzymes.

Digestion of the Enzyme by Other Enzymes.—The disintegration of the enzymes due to the attacks of other enzymes offers an interesting field. Euler and Josephson,[18] in attempting to digest sucrase by various proteoclastic enzymes, such as pepsin and trypsin, found that the former does not attack or weaken the sucrase and that the latter weakens it only slightly. The slight weakening in the early stages of its activity led them to the conclusion that the attack of the trypsin must be on the protein base to which the more active part of the enzyme is attached.

Is the Enzyme Gradually Destroyed as a Result of Its Activity?—Since the enzyme enters into the chemical reaction and then emerges apparently unchanged, a small amount of the enzyme will produce an amount of change which is out of all proportion to the amount used. Sucrase has the ability of inverting two hundred thousand times its weight of cane sugar[36] and Hammersten says that one part of rennet will clot four hundred thousand times its weight of caseinogen in milk. It would seem from these statements, however, that, in the course of time, the enzyme is gradually exhausted and worn out, or is acted upon by other destructive enzymes and, since it has no means of rebuilding from within, it finally fails to serve as a catalyst. When such minute quantities of enzymes are used, the length of time during which they must act must necessarily be increased, since their action is dependent upon the concentration of the enzyme.

The Rate of Enzyme Formation in Relation to Fungus Growth.—The secretion of diastase is dependent on the condition of the fungus and, since the liquefaction of the food must depend on the amount of enzyme, the process becomes more or less self-regulatory. Robbins[39] was unable to discover any particular chemical element necessary to diastase secretion in *P. camemberti*, neither potassium or calcium being intimately connected with its formation, or the same would appear to be true, too, of sulphur, chlorine, magnesium, and sodium, although Hawkins[24] had found that the chlorides in very weak concentrations accelerate malt-diastase digestion.

THE GENERAL PHYSIOLOGY OF ENZYMES

The General Principles of Enzyme Action and Composition.—After discussing the facts which have been discovered regarding sucrase, it may be well to notice some of the general principles of zymology that have been developed in the last 30 years' work in this field.[41,35,43,44] Even among the divergent theories all seem to agree at least on two points: (1) the enzymes act in a catalytic manner on the substance which they alter; (2) they are colloids.

1. The Enzymes as Catalysts.—Catalytic action is well known among inorganic compounds; platinum black is able to adsorb eight hundred times its volume of oxygen and then becomes an active oxidizing agent. A catalytic agent may be defined as a substance which enters into a chemical reaction but emerges unchanged. The enzymes are catalytic agents derived from living plants or animals which respond to external conditions in many ways, as do the organisms from which they are derived. They can be coagulated by heat or chemicals, such as strong alcohol, require water for their reactions, and deteriorate if kept any length of time. On the other hand, they are like chemicals in their reactions and in the manner in which these reactions are accelerated by temperature and concentration. A catalytic agent unites itself to the substance, connects it to a third substance, and the reaction having taken place, the catalytic agent is released unchanged. A catalytic agent increases the velocity of a reaction, that is to say, the reaction takes place more rapidly in its presence. This bridging action of the catalyst involves at once a dispute as to the nature of the union between the catalytic agent and the substances which it changes; is it a real chemical union or only an *adsorption* phenomenon?

The chemical theory of enzymes as catalytic agents is that a real molecular union occurs between the catalyst and the substance upon which it acts, a *chemosorption*, but the catalyst is freed from the combination as soon as the second substance influences the attachment through the formation of intermediate substances.[38] This viewpoint is not that of the majority of enzyme specialists.

The adsorption theory has met with much more favor from nearly all recent experimenters. The chemist describes as adsorption phenomena, those relations in which a substance applies itself to the outside of another substance without really entering into chemical union with it, the exact nature of this union is not definite, but the adsorbed substance is not held on the surface by a chemical bond of the ordinary sort. According to this view, enzyme action is primarily one of adsorption and the enzyme can act in liquids in which it is completely insoluble. The velocity of the reaction is dependent on the surfaces which may be brought into the adsorption reaction. The process is a physical one and is dependent on the form of the surfaces, since two surfaces which adsorb must be of such contour that they will come into intimate contact. It is possible that there is some chemical action where the surface of the enzyme comes into contact with the substratum upon which it acts, but this chemical action does not involve the whole enzyme.

The whole question of the method of action of the enzymes on their substratum, chemical or physical, has been studied by some of the most noted of the physical chemists, such as Arrhenius.

2. The Enzymes are Colloids.—All investigators agree that enzymes are colloidal in their nature, since they will not pass through a parchment membrane. They are particles too small to be seen by the ordinary microscope, but with an ultramicroscope they appear as shining little bodies which are in active Brownian movement. They are, therefore, to be regarded as entities, capable of coming within the range of our instruments and not, as are the molecules and atoms, lying beyond any of the ordinary methods of observation.

3. Are Enzymes Living or Only Chemicals?—The question arises at once whether enzymes are living bodies or simply chemicals. Buchner[7] believes them to be purly chemical since they are unable to assimilate and reproduce; a view probably correct, since it is difficult to imagine a living protein, which has been coagulated by alcohol, going again into solution with no loss of activity.[10]

Coagulation with alcohol is supposed to be, for most proteins, an irreversible reaction, but with the enzymes it does not seem to be so. On the other hand, precipitation by the use of heat kills the enzyme and coagulates the proteins. Two sorts of coagulation are possible here, a permanent reaction and a temporary one.[21]

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CHAPTER XVI

THE NATURAL FERMENTATION OF FRUIT JUICES

Unboiled fruit juices ferment from the action of the yeasts which have found their way into the expressed sap from the skin of the fruits. Fermenting juice under the microscope usually shows the predominant yeast to be *Saccharomyces apiculatus*, a very widespread variety in all soils and always present on fruits (Fig. 71).

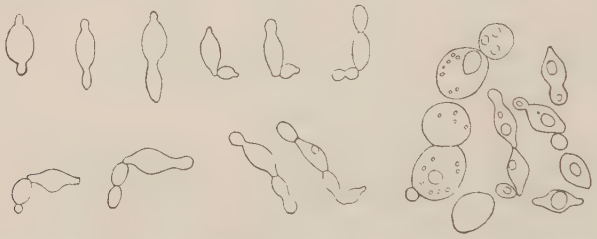

FIG. 71.—Budding cells of *S. apiculatus*. At right, some cells of *S. cerevisiæ* for comparison of sizes. × 600. (*After Hansen.*)

Effect of Growth of Saccharomyces Apiculatus.—The natural acidity of about 0.5 per cent in apple juice, largely from malic acid, and 0.45 to 0.85 per cent in grape juice, from the tartaric acid, prevents the growth of bacteria but does not injure the yeast.

Cider with a specific gravity about 1.050 contains about 9 to 10 per cent of sugar of which 7 to 8 per cent is reducing sugar and about 2 per cent cane sugar. The natural fermentation by the wild yeasts, the principal one being *S. apiculatus*, produces 4 to 6 per cent of acohol and the specific gravity is reduced to 1.0015, the acidity being slightly reduced in the process.[21] The Italian strains of this yeast produce between 3.15 and 4.55 per cent of alcohol.[27]

Müller-Thurgau[22] found that the seven races of this group isolated by him produced between 2.5 to 3.8 per cent by weight of alcohol, so that they only ferment a small part of the sugars. *S. apiculatus* is a yeast which requires oxygen and when this is supplied, the alcohol fermentation can be increased from 3.03 to 5.76 per cent, the fermenting power being almost doubled.

Good and Bad Effects in Wine Making.—Its powers of fermentation are further limited by the fact that many strains are unable to invert sugars, such as sucrose, owing to the absence of the enzyme, sucrase, their activities being limited to hexose sugars such as glucose and

fructose.[9] The special effect which some races of this yeast have on fermentation is in the production of sweet-smelling esters.[18] These fruity esters give a decided bouquet to wines in which better races are abundant; but, on the other hand, some races produce, instead of these desirable flavors, a musty and moldy taste. In addition to the production of these flavors, Müller-Thurgau[22] demonstrated that this yeast diminished the acidity of the fruit juice from 24 to 40 per cent of its original amount.

In general, this group is held to be a pest in wine making and various suggestions have been made to get rid of it by washing the fruit or by

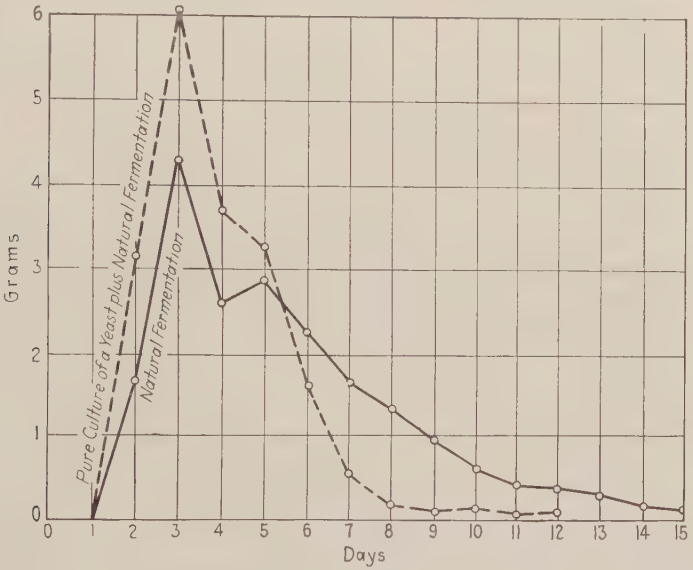

FIG. 72.—Grams of carbon dioxide lost in yeast fermentations. (*After Moncure and Ellett.*)

adding enough alcohol to the juice to inhibit its growth, since it is very susceptible to even 2 per cent of alcohol.

The course of the natural fermentation as indicated by the production of carbon dioxide (as the measure of the amount of alcohol produced) is shown in Fig. 72. It will be noticed that the greatest production of carbon dioxide occurs on the third day, and that there is a gradual decline on the following days, until the fermentation completes itself from the yeast being unable to tolerate the percentage of alcohol present. Since *S. apiculatus* will produce only about 4 to 6 per cent of alcohol, 1 to 2 per cent of sugar was still left after fermentation had ceased. This sweetens the cider, but if the alcohol is to be converted into vinegar, it is useless. By adding cultures of a yeast which is known to be a rapid fermenter, the production of carbon dioxide ceases more quickly.

Since the production of alcohol stops at about 5 per cent or a little less, with the ordinary yeasts, such as *S. apiculatus*, it is necessary, if all the sugar is to be converted in a fruit juice, such as that from very ripe grapes, often with 25 per cent of sugar,[2] that some other variety of yeast be added which is able to withstand a very high percentage of alcohol. Such a yeast is the *Saccharomyces ellipsoideus*, the true wine yeast of Europe which will produce up to 14 per cent of alcohol.

THE SACCHAROMYCES APICULATUS GROUP

The Organisms.—*Saccharomyces apiculatus* was at one time considered to be a single species but it has been found that species isolated from various soils differ in their fermentation of sugars; some will attack only glucose, fructose, and *d*-mannose, while others will ferment saccharose also. With the variation in fermenting power is associated some variation in the size of the cells in the different varieties.

The typical *S. apiculatus* has a lemon or ellipsoidal cell, 5 to 10 microns in length with sharp ends (Fig. 71). Budding occurs at the sharpened ends only, so that bud and mother cell are held together by a very small connection.

The various species have been described by Klöcker[12] and Rossi[27] and the literature reviewed by Klöcker.[13–15]

Varieties of this yeast are very abundant everywhere on ripening fruit and find their way into the juice where they take part in the normal fermentation. The name indicates that it is one of the true yeasts, but the endospore production is doubtful, although the attempt made by Lindner[13] led him to believe that a single spore is produced in each cell. This single spore may be only an accumulation of fat in the form of a large droplet in the cell. Klöcker[12] denies all spore formation and it has been removed from the true yeasts and renamed *Klöckeria*. The appearance of the fungus is so characteristic that it can be readily identified and Hansen[9] worked out the life history of wild yeasts first on this group.

THE SACCHAROMYCES ELLIPSOIDEUS GROUP

A number of varieties of wine yeast have been described so that *S. ellipsoideus*, instead of being a species' name, represents a group. The various developmental stages have been investigated by Kroemer and Elssmann.[17]

The Organisms.—The cells are spherical or oval bodies, about 8 to 10 microns in length, morphologically not very distinct from the domestic species. Endospores are formed, as in all the true yeasts, when they are deprived of food but are supplied with abundant air and moisture (Fig. 73). Young cells are almost homogeneous in appearance; older cells are filled with vacuoles and contain dark food granules; and the dead cells are very granular and almost opaque.

This variety of yeast does not seem to be indigenous to the United States since it was found only once by Holm[11] on the grapes he investigated in California, the common yeast being the omnipresent *S. apiculatus*. *S. ellipsoideus* is such a valuable species to have in a vineyard, however, that cultures of it have been obtained by many of the wine growers, and cultures of it, too, have been distributed for fermenting waste honey to make honey vinegar.[5] By the use of this yeast all of the sugar may be fermented unless there is an unusual percentage present in the grape juice or in the sugar solution. If the sugar is too abundant, a portion of it remains in the wine and the wine is sweet from its presence. *S. ellipsoideus* will form approximately 17 per cent of alcohol.

One or two per cent of sugar may be a source of danger in the wine, as it provides a food for the growth of bacteria which may develop bad flavors, colors, or a slimy or

stringy condition; *dry wines*, as the makers term them, keep much better than sweet wines.

Disinfecting Grape Juice.—The types of yeast found usually on the grapes produce only a very small amount of alcohol, about 1 or 2 per cent, so that they are really a detriment to the production of wine and should be destroyed in the grape juice and a better variety substituted. To destroy the wild yeasts and their allies, from 6 to 16 ounces of potassium metabisulphite, the usual amount being 12 to 16 ounces, is added to each 1,000 gallons of crushed grapes. After 12 to 24 hours, the organisms are all

FIG. 73.—The wine yeast, *S. ellipsoideus:* 1, young organisms; 2, old organisms; 3, endospores in the cells. (*After Bioletti.*)

dead or harmless, the juice is drawn off, and pure cultures of wine yeasts added. The small amount of sulphurous acid into which the potassium metabisulphite breaks, seems to do no damage to the fermentation which now begins.[24]

Amount of Alcohol from Various Percentages of Sugar.—The amount of alcohol and also of acetic acid which may be obtained, theoretically, from various percentages of sugar is shown in the following table:[3]

Sugar	Alcohol	Acetic acid	Sugar	Alcohol	Acetic acid
1.0	0.54	0.70	8.0	4.31	5.60
2.0	1.08	1.40	9.0	4.85	6.30
3.0	1.62	2.10	10.0	5.38	7.00
4.0	2.15	2.80	11.0	5.92	7.70
5.0	2.69	3.50	12.0	6.46	8.40
6.0	3.23	4.20	13.0	7.10	9.10
7.0	3.77	4.90	14.0	7.64	9.80
			15.0	8.18	10.50
			20.0	10.87	14.00
			25.0	13.56	17.50
			30.0	16.36	21.00

This table shows that, with a sugar content above 25 per cent, even *S. ellipsoideus* could not ferment it all and part of it would remain in the wine. The wine producer who has grapes with a sugar content above 25 per cent, therefore, mixes them with others that are not so sweet and thus gets a juice in which all the sugar will ferment. Some varieties of sweet wines are left with a percentage of sugar in them but such wines are very difficult to handle and keep.

THE LIFE HISTORY OF THE WILD YEASTS

We owe our fundamental knowledge of the life history of the wild yeasts to Hansen[9] who also contributed so much to the study of the ordinary domestic forms. Hansen worked out the life history of the common group of yeasts fermenting fruit juices, *Saccharomyces apiculatus*, in 1880 and 1881. This yeast grows on the ripe, juicy fruits which have been damaged, or cracked, so that the yeast can find its way into their interior. It increases here in great numbers, finding its way, of course, into the juice pressed from such fruit. It is not necessary, however, during the harvest season to have damaged fruit in order to induce so-called "spontaneous fermentation," for the yeast is also abundant in the soil of the orchard, having been washed there by the rains. The yeasts can be carried to other fruits by wind blowing dust laden with them; or, insects visiting the cracked grapes for the exuding sweet juice, become contaminated with yeasts and carry them to other uninoculated fruit. The wind, however, according to Hansen, is the principal agent in disseminating the yeasts, since the insects are to be found only after the fruit was ripe. A study of the soil in orchards and gardens showed that, while many of the true Saccharomycetes could be found at any season of the year, they were particularly abundant during the time that the sweet, juicy fruits were ripe, although they could live for several years in inoculated soil in flower pots.

Hansen's work was confirmed by Müller-Thurgau[23] who studied the Saccharomyces in the soil of the vineyards along the Rhine at Geisenheim. The yeast is confined to the upper 16 inches of soil, most of it in the first 8 to 12 inches where it can live from one vintage time to the next.

Wortmann[36] continued these studies on the yeasts in the soil of the vineyards of the Rhine valley at Geisenheim by counting the number of yeasts found every 2 weeks in typical samples. The largest numbers were found in November and December after the vintage was over, but while the soil was still thoroughly infected from the yeast which had grown on the grapes. The numbers dropped off during the winter and spring as the weaker ones succumbed to the lack of food, with a minimum in August and September, just before the grapes began to ripen. The great majority of yeasts in the soil seem to die during the winter, spring, and summer but a few live to carry the species into the autumn vintage season when they can again get food and multiply in numbers.

Hansen[9] later, extending his investigations to include not only the soil in orchards but that from distant meadows, found that while 67 per cent of the soil samples from orchards contained true Saccharomyces, only 30 per cent of the soils of adjacent forest fields show them, and only 19 per cent from distant fields. In mountain soils from the Alps and the Harz, the number was dependent on the distance from orchards or vineyards, indicating that fruit was the means by which they were perpetuated.

Yeasts are rather uncommon in the ordinary American soils.[31]

THE PINK, RED, BLACK, AND GRAY YEASTS

If a Petri dish filled with an agar containing nutrient salts and some form of sugar is exposed to the air, out doors, in the laboratory, or in the home, numerous mold colonies and some smaller, dull, waxy colonies of various colors will develop in about a week. The latter colonies, showing none of the light cottony growth to be found on molds, are wild yeasts. They are abundant everywhere in nature in the air.[7,8] They are found in maple sap coming from the bark on the trees and, finding their way into the sap buckets, produce changes[4] detrimental to the quality of the syrup and sugar. A red color in sauerkraut may be produced by their presence.[6]

The yeast-like forms which occur in the air are mostly Torulas (spherical types) according to the very complete studies which Saito and Naganishi[29] have made of

them in the atmosphere of Japan. These investigators exposed Petri dishes six times every month, in a variety of locations, in order to determine the distribution and relative number, and found them uncommon over open fields; more abundant in cold or dry weather, and on windy days.

Under the compound microscope the small cells are found usually to be spherical. Reproduction is by budding but a few of them produce endospores. We really know very little about the life history or the relationship of these yeasts. The only work done on them in recent years is a description of the species isolated.[30,33] Many of them are undoubtedly stages in the life history of other fungi with relationships not yet determined.[34]

Pasteur[25] examined these yeasts with care in his study of the organisms occurring in beer, but as his work was done before the days of pure cultures, it is doubtful if he had pure strains. He found a number of varieties present, most of them being small spherical cells which might change into more elongated forms but which never formed a mycelium. His name "Torula" has been preserved for the spherical wild yeasts, although their relationship to the imperfect fungi causes more recent workers to put them with this group.

Hansen[9] contributed more than any other investigator to the classification of the budding fungi when he discovered that many of them form endospores in culture, while others do not.

On this basis, he divided them into two groups, the *Torulaceæ*, and the *Saccharomycetes*, the latter term being applied to the species which form endospores, the true yeasts. The Torulas were characterized by their spherical cells and lack of endospores. In addition to these two groups, however, it must be remembered that there are numerous Hyphomycetes to be found in cultures from the soil or air. Some of the wild Torulas, while they will not ferment sugar nor produce endospores, are oval in shape or even quite elongated.[33] Very few of the Torulaceæ set up fermentations in sugar to produce alcohol and carbon dioxide.

Composition of the Coloring Matter.—Color has been made the basis of classification in these wild yeasts but some of the normally gray yeasts, such as *S. ellipsoideus*, become pink if some of the salts of magnesium are in the culture medium.[16,33] The percentage of this element necessary may be very small but without it the colonies are gray. The coloring matter is one of the carotins, possibly carotin itself, at times.[30] The pink coloring matter is inside the cells themselves and can be extracted only by rubbing a culture with sand, breaking up the cell walls. Carbon disulphid absorbs it to form a dark red liquid. Light retards the development of coloring matter.

THE MYCODERMAS

The surface of pickle vats exposed to the air often becomes covered with a white scum which, if allowed to continue to grow, will render the pickles no longer edible, owing to their lack of flavor. The organisms seem to convert the acetic acid into an insipid liquid. The fact that they grow on the surface of liquids and never in their depths has given them their name, *Mycoderma* (myco = fungus, derma = skin), indicating that they are very dependent on oxygen for their existence. The viewpoint is not entirely correct, however, since the surface growth may be due[20] to the lifting power of the gases held in the complicated film aggregations which the fungus forms. The plants themselves are actually heavier than the liquid in which they are found, but the gases in their intercellular spaces buoy them up.

The fungus shows the greatest variation in the size, shape, and arrangement of the cells so that it seems to represent, not one, but many different fungi (Fig. 74). The buds, instead of dropping off after they have grown to full size, frequently remain attached, and a long row of them may form a pseudomycelium with numerous branches

on all sides. In other places, the cells seem to fall apart as soon as the bud is fully formed and the cells are nearly all single or in pairs, many of them with oil droplets. It is claimed by Winogradsky[35] that these variations are the results of larger or smaller quantities of oxygen in the different parts of the culture medium. If the oxygen is abundant, the majority of the cells will be yeast-like in character, but, in the depths of the culture where oxygen is scarcer, they will grow into a mycelium. In spite of these variations in form and arrangement, Meissner claims that it is possible to identify the different races of Mycoderma by size and shape of the cells and their rate of reproduction in cultures. The characteristics of the superficial vegetation are also different in the different races, and the changes which they produce in the substratum upon which they live indicate the peculiarities of the strains.

These organisms are of importance in the brewing and distilling industries where they may grow in the surface of the fermented or partly fermented juices and cause considerable damage.

FIG. 74.—Mycoderma sp. × 350.

The important physiological fact connected with them is their destruction of organic acids and alcohol. They may destroy only the organic acids such as lactic, malic, and acetic, but they may also form other acids from them, dependent on the race of Mycoderma present, and on the amount of oxygen supplied. Formic and butyric acids are believed to be among these products but in addition there are often fixed acids and esters. This would mean, under certain conditions, no diminution in acidity, but with the forms which usually occur on vinegar pickles, the lack of acidity is notable after Mycoderma has grown on them. With ammonium phosphate and chloride and the other necessary nutrient salts, the fungus can utilize alcohol if this is added as a source of energy, the alcohol being used partly for respiration and partly for the building of the fungus cells.

PATHOGENIC YEASTS

A few organisms with yeast-like forms are found to be pathogenic to man, causing some of the rarer skin diseases. Such a disease was first described from Baltimore by Gilchrist and later studied in detail by Ricketts.[26] The organisms seem to be of the yeast type but no endospores have been described, so that it must be regarded as one of the false yeasts, according to Hansen's interpretation. It buds, however, like a true yeast, although it seems to form at times a few hyphae. Yeasts and yeast-like fungi may occur in the digestive tract,[1] and the

serological institute in Vienna has isolated a number of pathological forms of yeasts which have been studied as to their biological characters by Malowan;[19] others in Japan by Sasakawa.[28]

THE RELATIONSHIPS OF YEASTS TO OTHER FUNGI

The constancy of the species of the yeasts has been implied in all the work that has been done upon them in recent years. They are regarded by most students of the group as a separate and distinct branch of the Ascomycete fungi, owing to the fact that they produce typically four or eight endospores. The typical ascus contains eight ascospores so that according to this theory, *the yeast cells would represent only asci*, the vegetative mycelium having completely degenerated. For this reason, the yeasts are usually considered to be degenerate Ascomycetes.

Brefeld believed at one time that yeasts were the conidial stage of some of the smuts. The conidia of the smuts in culture solutions bud in typical yeast-like manner, and may be kept growing as yeasts in pure culture indefinitely. Brefeld noted that yeasts were especially abundant in his cultures when he had the smuts around his laboratory, so he came to the conclusion that the yeasts were nothing but degenerated smuts which had adapted themselves to budding reproduction. He later changed to the more generally accepted viewpoint that yeasts are a primitive form of the Ascomycetes, a side branch which never developed any further in the evolution of the fungi.[32]

Many varieties of the gray, red, and black yeasts are only degenerate, budding stages of the Imperfect Fungi and should not be classed with the yeasts; they should be distributed through this large and heterogeneous group.

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CHAPTER XVII

THEORIES TO EXPLAIN THE PRODUCTION OF ALCOHOL

The high percentage of alcohol produced by the yeast plants when grown in sugar solutions would seem to need some adequate explanation and a number of ingenious theories have been proposed. The problem is both chemical and biological in its nature.

The great chemist (Liebig) who first answered the question gave a purely chemical and wrong answer. The second chemist (Pasteur) who attempted it gave a chemical-biological answer which was, in part at least, correct, but which has been modified. From the long controversy between these men and their followers, science has added many facts about yeast and fungus nutrition and about the general question of fermentation and respiration.

Liebig's Theory.—In sugar solutions, the nitrogenous ferments attack the very complex and unstable sugar molecule and break it down into simpler and more stable forms, such as carbon dioxide and alcohol. After the destruction has begun, it will continue without further help of the ferments. These ferments are not of definite composition but any nitrogen-containing body can start it if in contact with a body of complex constitution which is capable of fermentation. The yeasts contain nitrogenous substances which are easily destroyed and which work as ferments, but the fermentation has nothing whatsoever to do with life activities of the yeast plant itself.[9]

Pasteur's Theory.—Liebig's theory was a purely chemical one while Pasteur's was a vital one, using this term in the sense that the fermentation was the result of the vital activities of the yeast plants as they grew in the culture solution. Without the activity of the living yeast, fermentation would be impossible, since it is by the destruction of the sugar into alcohol and carbon dioxide that the yeast gets its food and energy. The sugar is destroyed in the entire absence of oxygen, *i.e.* the process is termed an *anærobic* one, the yeast plants respiring and getting energy and food without the help of the oxygen of the air. Since the process should take place in the absence of air, the admission of air stops or retards it.

Later Theories.—It is of interest that in the main the Pasteur theory has been discarded just as has the earlier one of Liebig. Brefeld[1] showed that the fermentation would go on just the same in a sugar solution if oxygen were admitted freely, an observation confirmed by Buchner

and Rapp.[2] The fact that the yeast cell was not necessary for the fermentation process but that an enzyme could be extracted from the cell which would carry on the chemical change just as well was shown by Buchner, Buchner, and Hahn[3] in a long set of experiments, which has removed the essential support of Pasteur's notion, although yeast is necessary for the production of the enzymes involved. The chemical changes which are induced by this enzyme, however, are complex and no general agreement can be reached among the chemists who have attacked the problem. We still hold to the vitalistic theory but only with the reservation that the substances which bring about the reactions are enzymes, not the yeast cells themselves.

THEORIES TO EXPLAIN THE HIGH PERCENTAGE OF ALCOHOL

Kostychev[5] has suggested that the formation of alcohol in the yeast plants is due to the imperfect oxidation of this product of cell activity; alcohol is one of the oxidation by-products of the yeast respiration, the oxidation of which is incomplete. Alcohol has been found in the higher plants in small quantities when the oxidation process did not keep pace with the formation of the intermediate product, alcohol. If it is complete, the sugar will be wholly converted into CO_2 and H_2O. The alcohol fermentation is to be regarded as only an intermediate stage of normal respiration.

The two processes of respiration and fermentation can be represented as follows:

$$\text{Respiration} \quad C_6H_{12}O_6 + 6CO_2 = 6CO_2 + 6H_2O$$
$$\text{Fermentation} \quad C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

It will be seen from this equation that fermentation is, as Pasteur described it, "life without oxygen."

Kostychev,[5] following up this theory, investigated CO_2 and alcohol formation in some of the common mushrooms, such as *A. campestris*, and in the mold *A. niger*. Carbon-dioxide production is very vigorous in the young mushrooms but no alcohol seems to be produced. *A. niger*, when grown in media without a carbohydrate, will still produce carbon dioxide but no alcohol, so anærobic fermentation does not necessarily mean the production of alcohol. If the mycelium is covered by the culture medium,[8] however, both carbon dioxide and alcohol are formed even in a culture solution containing no sugar, but it is very weak in both cases even under these anærobic conditions.[6,7]

Iwanowsky[4] suggested that the production of alcohol was the result of unbalanced culture solutions, the sugar being abnormally high and the peptone low. The yeast cells in order to dispose of the sugar in some manner convert it into alcohol and excrete it. This process is analogous to the destruction of proteins in the body of plants or animals where amides or amido acids are formed. The amides that are formed are often collected in the cell sap. In the sugar fermentation the by-products are stored not in the cell sap nor in the body of the organisms but are passed out into the surrounding liquid.

Iwanowsky grew yeast in broad-bottomed flasks which gave the plants an access to an abundance of oxygen. The gases over the liquid were measured for carbon dioxide and oxygen, in order to determine the ratio of the carbon dioxide produced to the oxygen consumed from the air. Part of the carbon dioxide would be produced in the destruction of the sugar, the oxygen entering this portion being derived not from the air but from the sugar molecule. If the sugar is greatly in excess, the ratio is $\frac{CO_2}{O_2} = 18.4$, but with the sugar percentage lowered and the peptone increased, the ratio falls to 2.0, 1.8, and even to 1.2. In these latter cases, the carbon dioxide production is to be regarded as the normal respiration of the yeast plants, the oxidation of the waste materials of the organism. Where a minimum of sugar was added, only 0.10 to 0.15 gram in 50 cubic centimeters of the culture solution, the sugar disappeared long before the end of his experiment, which usually lasted 2 to 3 days.

Iwanowsky's interesting theory that the yeast fermentation is only a pathological condition produced in the yeast cells was subjected to criticism by Richter,[10] who conducted experiments in the same manner as Iwanowsky but secured different data. Using a culture solution with the following components:

50 cubic centimeters nutrient salt solution
(K_2HPO_4 and $MgSO_4$, with a trace of iron)
0.15 gram cane sugar
0.25 gram peptone

in a hermetically sealed flask with a large bottom, he obtained the following results:

	After 24 Hours	After 48 Hours	After 96 Hours
$\frac{CO_2}{O_2}$	4.26	2.25	1.86

But by increasing the percentage of sugar and peptone so that the solution contained:

50 cubic centimeters nutrient salt solution
0.3 gram cane sugar
0.5 gram peptone

he obtained the following proportions for the carbon dioxide and oxygen:

	After 24 Hours	After 48 Hours
$\frac{CO_2}{O_2}$	8.32	6.16

While if the sugar and peptone content of the solution were further increased to the following:

50 cubic centimeters nutrient salt solution
0.75 gram cane sugar
1.25 grams peptone

the relations were as follows:

	After 24 Hours	After 48 Hours
$\frac{CO_2}{O_2}$	11.16	27.46

The conclusion which he draws from these data is that yeast cells prefer the cane sugar as a source of carbon, and if furnished with it will use it even with an abundance of peptone. The sugar fermentation is a natural one for the yeast cells and is not the result of pathological conditions induced by an excessive sugar content of the culture medium.

No Satisfactory Explanation.—No completely satisfactory explanation for the high production of alcohol by some varieties of yeasts has been offered as yet. Some varieties form it, others do not. Some seem to be adapted to alcohol formation and tolerance. The theory

of Kostychev seems to offer as sound a basis as any that has been proposed: alcohol is formed as the product of incomplete respiration; it is an intermediate product.

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CHAPTER XVIII

A GENERAL ACCOUNT OF THE BACTERIA

Morphology of the Cells.—The cells of the bacteria vary in form and size. The four type forms are spherical, rod, corkscrew, and thread

The spherical forms, (*coccus*) (Fig. 75), may be slightly elongated and indistinguishable from rods. If one diameter is slightly greater than the other two, the organism is considered to belong to the rod-like group (*bacillus* or *bacterium*). The corkscrew-like group (*spirillum*)

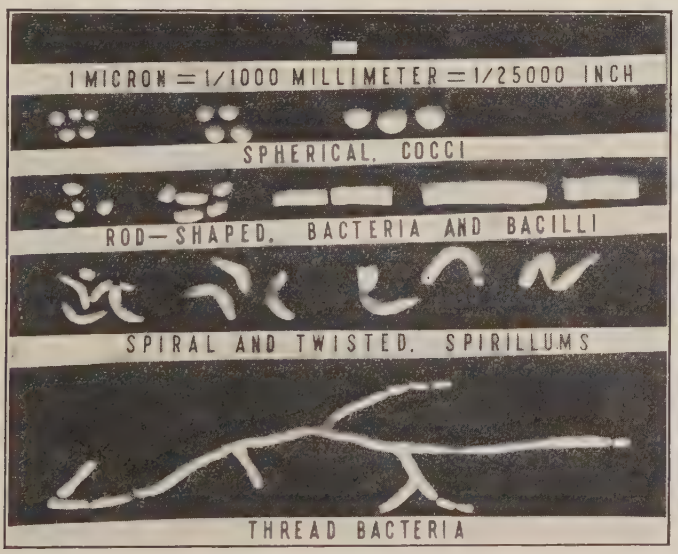
1 MICRON = 1/1000 MILLIMETER = 1/25000 INCH
SPHERICAL. COCCI
ROD—SHAPED. BACTERIA AND BACILLI
SPIRAL AND TWISTED. SPIRILLUMS
THREAD BACTERIA

FIG. 75.—Bacterial shapes: coccus species are *Micrococcus minimus*, *M. urea*, *M. aurantiaca;* rod-shaped species are *Bacillus prodigiosus*, *B. typhosus*, *B. subtilis*, and *B. anthracis;* spiral species are *Vibrio percolans*, *V. cholereae*, and *Spirillum rubrum;* thread bacterium is *Actinomyces sp.* (*Measurements according to Bergey.*)

includes all of the rods which are twisted, the twist varying from comma-like to an elaborate corkscrew of two or more turns. The thread-like organisms (Actinomyces, Thiothrix, etc.) vary in the size and branching of the cells but are more elongated than any of the rods.

Structure of the Cell.—The small size of the cells makes their accurate study difficult. The very thin cell wall encloses the protoplasm. The composition of this wall is difficult to determine, but the presence of both chitin and cellulose is probable, and it is sometimes impregnated with fatty compounds.

The protoplasm does not contain a nucleus, according to the older authors, but the more recent viewpoint is that the nucleus, instead of being concentrated into a single sphere, is composed of a variable number of granules irregularly diffused through the cytoplasm. Some investigators claim that these granules, at times, aggregate to produce a definite body which corresponds to the nucleus of higher plants and animals. In addition to these chromatin granules, food particles, both carbohydrate and protein, appear, scattered in the cytoplasm; at times, fat droplets and, in some species, sulphur bodies can be seen.

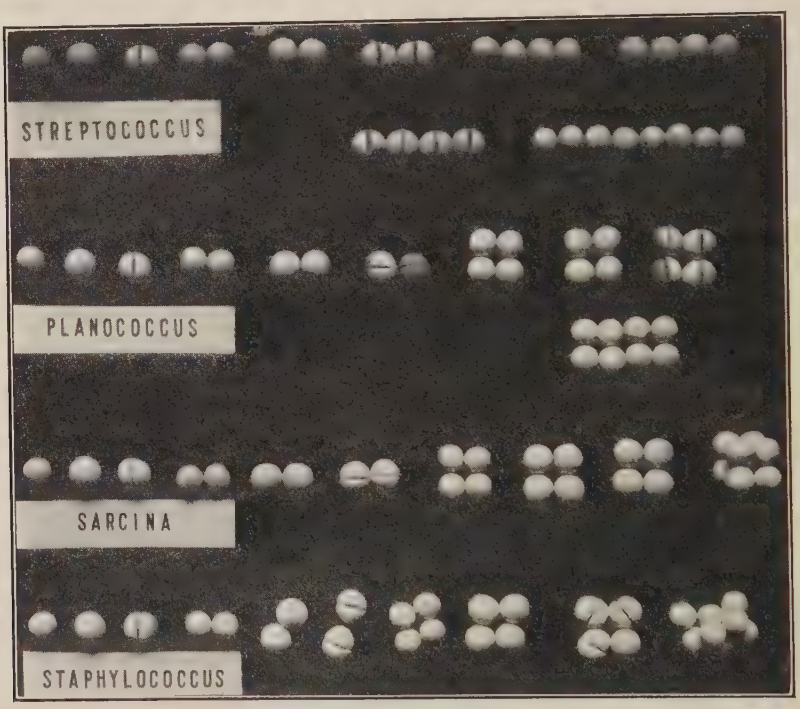

FIG. 76.—Effect of planes of division on grouping.

Size of the Cells.—The majority of the spherical (*coccus*) species are small, ½ to 1μ in diameter, but larger and, also, smaller species occur. The rod-like species are usually somewhat larger, ½ to 1μ in length, while the corkscrew-like types are often much longer, and the thread types may be hundreds of microns in length.

Reproduction in the Bacteria.—A cell lengthens and a cell wall is formed across its middle, cutting it into new individuals (Fig. 76). This wall splits into two and the new, daughter cells may remain adhering or they may at once fall apart. The process is known as *fission* and gives this group the name, *fission fungi* or *Schizomycetes*.

If division in the cocci is in only one plane, a chain is produced (Fig. 76), *Streptococcus*. If in two planes at right angles to each other, a plate of bacteria one cell in thickness, *Planococcus* or *Micrococcus*, results. If in three planes at right angles, an approximately equal number of divisions in each, a packet, *Sarcina*, is formed. Irregular grape-like aggregations, the result of divisions in three planes irregularly placed with reference to each other is *Staphylococcus*. Groups of two are called *Diplococcus*.

Division in the rod- and screw-like species is preceded by cell lengthening without any appreciable increase in diameter. The daughter cells may hang together, end to end, to form chains of two or more individuals, or at times, long threads.

False branching may occur in some of the thread species when the cells divide, one of the daughter cells slipping to one side. True branching also occurs by the formation of side outgrowths. Reproduction seems to be largely by fragmentation or by the formation of conidia somewhat similar to those of molds.

Movement in the Bacteria.—Many species of the rod- and cork-screw-like types are motile; only a few of the cocci and none of the thread-like organisms move. Movement is effected by the lashing of protrusions from the body. These organs (*flagella* or *cilia*) cannot be seen under the ordinary illumination and special staining methods are required to make them visible. They are very delicate colloidal structures, very active in the young individual but lost as it ages. The number and point of attachment of the flagella vary in the different species; in some, a single flagellum at one end; in others, a group of flagella; while, in others, the entire cell is covered with them. These protrusions are often longer than the cell itself, but may be shortened or lengthened.

Resting Spores in the Bacteria.—Inside the cells of some species are formed small, refractive bodies (*spores* or *endospores*); one spore to each cell. These spores being very resistant to heat, steam, chemicals, drying, etc., carry the species over periods unfavorable to growth. They are provided with very thick impermeable walls, and the contents probably contain very little water.

The thick walls prevent the entrance of water or other liquids into the cell and also prevent the small residuum of water inside them from escaping. The very low water content of the protoplasm prevents its easy coagulation, coagulation being the visible evidence of death.

In certain other species, the contents of the cell break into smaller bodies (*gonidia*); these do not seem to have the resistant properties of true spores but do grow into bacteria.

Only one spore is formed in a cell, so spore formation does not increase the number of bacteria.

The spores germinate by the absorption of water, the splitting of the very dense wall, and the issuing forth of one of the *vegetative* cells.

The rate of increase in numbers is dependent on food and temperature. The food must be of a type adapted to the organism. These requirements vary widely but bacteria may be divided into those requiring only inorganic chemicals, *prototrophic*, (proto = primitive, trophic = food); those to which organic compounds, especially proteins from meat or blood, are essential, *metatrophic*, (meta = middle, trophic = food); those living only in the bodies of plants or animals and deriving their food from them, *paratrophic*, (para = upon, trophic = food), *i.e.* parasitic.

Every organism has a temperature at which it will grow best, cell divisions occurring at the shortest intervals. At warmer and colder temperatures than this *optimum*, the growth, while it continues, is at a slower rate, and the time lengthens between divisions. This slowing continues until a temperature (high or *maximum;* low or *minimum*) is reached where growth just about ceases. Beyond these points lies the thermal death point especially that above the maximum temperature. For the ordinary bacteria, the optimum temperature lies between 25 and 38°C. while the thermal death point is between 52 and 65°C. At the optimum temperature, the length of life of one generation may be only 20 minutes, while a lowering of the temperature may increase it to 12 hours. Bacteria have been divided into the following temperature groups with minimum, optimum, and maximum temperatures indicated:

1. *Psychrophylic* or cryophylic (0 to 10°; — 20°; — 30°).
2. *Mesophylic* (10 to 15°; — 37°; — 45°).
3. *Thermophylic* (40 to 50°; — 50 to 55°; — 60 to 72°).

The majority of organisms are in the mesophylic group.

Relation of Bacteria to Oxygen.—Some bacteria can grow only in situations where an abundance of oxygen can be obtained, *ærobes;* others live only in the absence of oxygen, *anærobes*. Between the two extremes are species which can live in the air or can adapt themselves to a minimum of it, the *facultative anærobes;* or, which live best without air but can still grow in it, *facultative ærobes*. The ærobes require oxygen for respiration, for the oxidation of carbon compounds; the anærobes respire by rearranging carbon compounds so that CO_2 appears as a product.

Secretion of Enzymes.—Some species of bacteria liquefy gelatin; others do not. This bacterial activity was at one time considered to be very stable and was used in describing all species. The liquefaction occurs through the action of proteoclastic enzymes.

Milk may be coagulated by rennet-like enzymes secreted by some of the bacteria, the curd being afterwards digested by proteoclastic ones.

The sugars such as sucrose, are destroyed by inverting enzymes, into invert sugars. Other enzymes break hexose sugars into CO_2 and water or CO_2 and H_2. Milk sugar may be split into lactic acid.

Relation to Acid and Alkali.—Bacteria thrive best in a neutral or slightly alkaline medium. The addition of a small per cent of either acid or alkali checks growth.

Staining Reactions of Bacteria.—The cells of bacteria, coagulated by heat or chemicals, take up and retain the anilin (aqueous soluble) stains very readily. Some bacteria retain these stains against the bleaching action of acid alcohol and are called *acid fast*. Others retain a gentian-violet-iodine stain against decolorization by alcohol and are *gram positive*. If they lose it, they are *gram negative*. These staining reactions are of considerable value in describing species.

The Classification of the Bacteria.—Since the bacteria are among the most important groups to be studied in a course in microbiology, the following simple classification, based in general on that of Migula[3] with certain modifications adopted by Lieske,[2] will be sufficient to place the principal groups, as they have been arranged largely for practical purposes. Although the system may not be entirely accurate or complete in all details, we must have some common basis upon which to discuss the organisms as classes of bacteria, and this classification is in general use. It is like a common foreign language in which scientists can, to some extent, understand each other, although the meaning may be, at times, a little vague. The Migula system is based on still older systems of classification, but the basis of all of them is the morphology of the organisms with resort to their physiology, if the morphology gives no means of identification. The *genera* are separated from each other on the basis of their shape, and the *species* on their physiology. The principal groups of bacteria are the following:

1. The Coccaceæ, spherical organisms.
   1. Streptococcus, organisms in chains, divisions all in one plane.
   2. Micrococcus, organisms in plates of one coccus in thickness, divisions in two planes.
   3. Sarcina, organisms in regular packets, divisions in three planes.
2. The Bacteriaceæ, rod-shaped organisms.
   1. Bacterium, cells with endospores, not motile.
   2. Bacillus, cells with endospores, motile by flagella placed all around the body.
   3. Pseudomonas, cells occasionally with endospores, flagella only at the poles.
3. Spirillaceæ, spiral or twisted organisms.
   1. Vibrio, cells which are only slightly twisted.
   2. Spirillum, cells are corkscrew-shaped.
4. Mycobacteriaceæ, thread-shaped bacteria.
5. Chlamydobacteriaceæ, cells arranged in chains and surrounded by a sheath.
6. Rhodobacteriaceæ, cells contain a red pigment and sulphur.
7. Thiobacteriaceæ, cells contain sulphur but no coloring matter.
8. Myxobacteriaceæ, cells swarm into a plasmodium at one stage in their life history, and form more or less elaborate fruiting bodies.
9. Spirochetaceæ, cells are corkscrew-shaped but move like a corkscrew or snake without the help of any observable flagella.

In addition to the above groups of the bacteria, the literature of bacteriology still retains certain other names such as *Diplococcus* for two spherical organisms which remain united. They may be regarded as short chains. The word *Staphylococcus* is used to designate a grouping of spherical organisms into a mass similar to that of a bunch of grapes. Such a mass arises by divisions in three planes irregularly disposed with reference to each other.

The principal groups of the bacteria have been limited and defined, historically, in the excellent book by Buchanan.[1] Usuage, even in technical literature, does not often follow the historically correct name.

Bacteria and Bacilli as Popular Names.—Several of the names which will be used extensively later, have two meanings and uses; one, a popular or common; the other, a scientific one. Words such as *bacteria* and *bacilli* are used as synonyms, in popular language, for *germs*, *microbes*, or *microorganisms*. In such usage they signify only minute organisms. Care must be used in the use of these words and in noting the sense in which they are applied.

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CHAPTER XIX

BACILLUS SUBTILIS

(THE HAY BACILLUS)

This organism is a widespread species found everywhere in the upper layers of the soil and in the dust on hay or grass. Its spores, being resistant to heat and drought, suggested to Roberts the following method for its isolation:[36]

Dry hay is chopped fine, covered with water for about 4 hours; the acid water is decanted off, neutralized to litmus, diluted to a specific gravity of 1.004, and boiled for an hour over a sand bath in a flask plugged with cotton. If the liquor was a little more than a liter at the beginning of the boiling, at least half a liter should remain in the flask. This remaining liquor is incubated at a temperature of 25°C. for 36 hours when a scum of white bacteria should appear on the surface from the spores which have escaped the hour's boiling. The organisms, if examined under the microscope, will show long chains of rod bacteria, the chains being arranged roughly parallel to each other. If the material is taken out with a needle and spread on a slide, the appearance will be lost, so that the best way to study the arrangement is to drop a clean cover glass on the surface film after the liquid has been carefully poured into another dish.

Cohn[12] repeated Roberts' experiments and found that with an acid hay infusion the sterilization was easy, but that if he neutralized it, or made it alkaline, a single boiling did not sterilize it. If kept for 12 to 20 hours, however, any of the solutions, regardless of whether they had been neutralized or not, became cloudy from the growth of bacteria, and a layer of bacteria gathered on the surface of the liquid.

Roberts' method gives cultures of members of the *B. subtilis* group but these must be purified by the plating-out method. On Petri dishes incubated at a temperature of about 25°C. for 3 or 4 days, dry, white, somewhat wrinkled, circular colonies about ½ centimeter in width will appear.[9,27]

Transfers from these plates give an abundant growth in sterile beef broth in 24 hours, the bacteria appearing largely on the surface of the liquid as a white scum. An examination of these rod-shaped bacilli under the microscope reveals the fact that they are in active motion. This motion is not the Brownian movement, such as is shown by inorganic particles in a liquid, but is an active, forward progress of the cells in a zig-zag path with no visible organs of locomotion.

The specific gravity of the bacillus is slightly heavier than that of water; 1.120 in 80-day old cultures and 1 134 in 40-day cultures, a difference sufficient to make them fall to the bottom of a broth culture if they have been growing on top.[38]

A number of varieties of the *B. subtilis* might be isolated by these methods but it will be assumed that a typical species, such as that described by Cohn, will be selected for the following studies (Figs. 77 and 78).

The Structure of the Cell.—The small size of these organisms makes any study of the wall and of the bacterial contents very difficult and almost impossible. The wall of the bacteria was believed to be one of the principal reasons for connecting up

these organisms with the plants and was supposed to be similar in chemical composition to fungus cellulose, a carbohydrate impregnated with some compound such as chitin. Although Van Wisselingh was unable to detect chitin in the bacteria, Veihoever,[40] by carefully heating his bacterial preparations in the autoclave at 160°C. for 15 minutes with a 50 per cent solution of potassium hydroxide, found that the spore walls, the sporangia (the bacterial cells which contain spores), and the remains of the old wall gave the characteristic violet reaction of *myosin* (*chitosan*) with the iodine-potassium iodide solution (2 grams of iodine, 1 gram of potassium iodide in 200 cubic centimeters of water) and with 1 per cent sulphuric acid. Very probably therefore, the cell wall is composed, at least in part, of chitin. Tests for cellulose seem to be entirely negative.

The cell contents show no structure except a few granules of stored food, and the protoplasm does not seem to be differentiated into cytoplasm and nucleus, as occurs

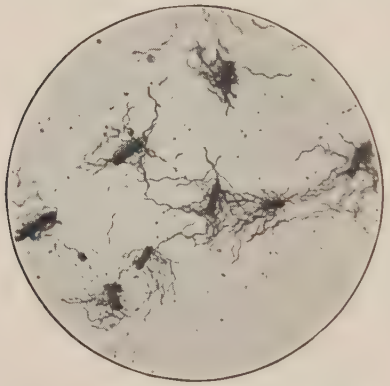


FIG. 77.—*Bacillus subtilis*, with flagella stained. (*From Kral Institute.*)

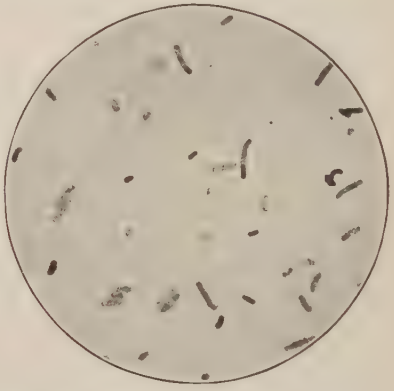


FIG. 78.—*Bacillus subtilis*, with spores. (*From Kral Institute.*)

in higher plants and animals. The older authors made the definite statement that bacteria were entirely without nuclei, but the matter is a disputed one and will be discussed later in connection with larger bacteria. The ordinary preparations, fixed in the flame and stained with some of the anilin dyes, certainly show nothing inside the protoplasm, but these methods are very crude for such small structures.

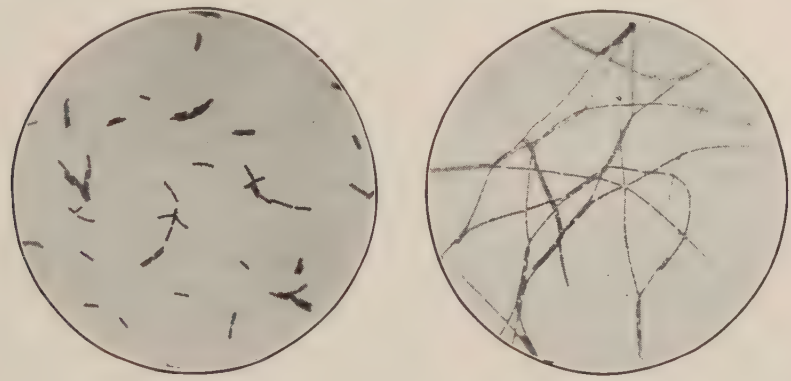
The size of the organism varies within certain large limits. The measurements given for this bacillus, when grown on the ordinary meat extract-peptone media, are, length, 3 to 4 microns and breadth, 0.8 micron; the longer individuals being ready to divide into two.[25]

Buchner[5] noted great variations in the size, particularly the length, of the cells of the hay bacillus, due to the chemical composition of the substratum upon which the organisms are grown: whether neutral, acid, or alkaline in reaction; whether a hay infusion made from the leaves or the stems of the plants; whether it contains sugar or not; and whether iodine has been added to the culture media. Excluding the iodine experiments, the general principle seems to be clear that the cells which are not well nourished are long and slender, while those which have been well fed are shorter and broader.

Buchner's results were from cultures whose purity of species might be questioned, as his observations were being made just at the beginning of the pure culture era, but they were confirmed in general by Fischer,[16] who undoubtedly had pure strains.

Many further studies, upon the amount of variation possible to induce in an organism as the result of the medium upon which it is grown, are being made. An organism seems to have an approximate given size and proportion only when grown at a specified temperature in a given medium containing definite percentages of nutrients of a certain acidity or alkalinity. Growth in ordinary culture media gives a very distorted view. Only a small part of the cycle appears in a culture at any one time and the conditions are very different from those which occur under natural conditions. The short forms which we know as "bacteria" may be only a type of spore termed, by Meyer,[31] an *oidium* (plural, *oidia*) implying that it is a fragment, the remains of a larger organism.

Dissociation.—Soule[37] isolated, from a culture brought in 1888 from Koch's laboratory, three strains of *B. subtilis*, which he designated as *S*, *R*, and *P* types (Fig. 79). The *S* type corresponds to the old form described by Cohn but the *R* type



S *R*

FIG. 79.—Two strains of *B. subtilis* isolated from original Koch culture: *S*, largely single motile cells; *R*, cells in long strands and non-motile. (*Photo by Soule.*)

is not motile and forms chains (Fig. 79). The *P* type is very rare with almost translucent colonies very difficult to see. The *S* and *R* strains are identical biochemically. The *R* form seems to be very stable but the *S* form continues to break up into *R* and *S* types. The motile *S* type is the old *B. subtilis* of Cohn, and the descriptions given here apply only to it.

Rate of Growth at Various Temperatures.—The rapidity of division in *B. subtilis*, or any other organism, is dependent to a considerable extent on the temperature in which it is placed. In *B. subtilis*, for example, Brefeld[3] saw the organism grow to full length and divide in ½ hour at 30°C.; in ¾ hour at 25°C.; in 1½ hours at 20°C. Division and growth practically cease at 7°C. or if the temperature is increased to 45°C. *B. subtilis* adapts itself in the soil to a fairly cool temperature, remaining in a resting condition during the winter months.

THE INCREASE IN NUMBERS IN A BACTERIAL CULTURE

Cohn calculated that an organism 2 microns in length and 1 micron in breadth (about the length but a little larger in breadth than the usual *B. subtilis* cell) would weigh 0.000000001571 milligram and would divide once every hour. In 2 days' time, the descendents of this single organism would number 281,000,000,000 and would occupy a volume of about ½ liter; while in 3 days, its mass would be sufficient to occupy the beds of all the oceans and its numbers would be expressed in thirty-seven places of figures. Fortunately, no such condition could ever occur, as the

bacteria in a culture retard each other's growth by their own excretion products, even if food is still abundant.

Cohn's figures based on a straight geometrical increase are not correct. If a transfer is made into a tube of fresh culture media, the numbers which appear in the succeeding hours follow a definite series of additions. Lane-Clayton[26] determined the number of living, growing organisms after different periods, starting with a transfer to fresh media, and came to the conclusion that the life cycle in a culture could be divided into four phases: (1) *the latent period*, the initial phase of slow growth or no growth at all; (2) *the period of maximum rate of growth*, the logarithmic period when the organisms were increasing in numbers in geometrical progression; (3) *the stationary*

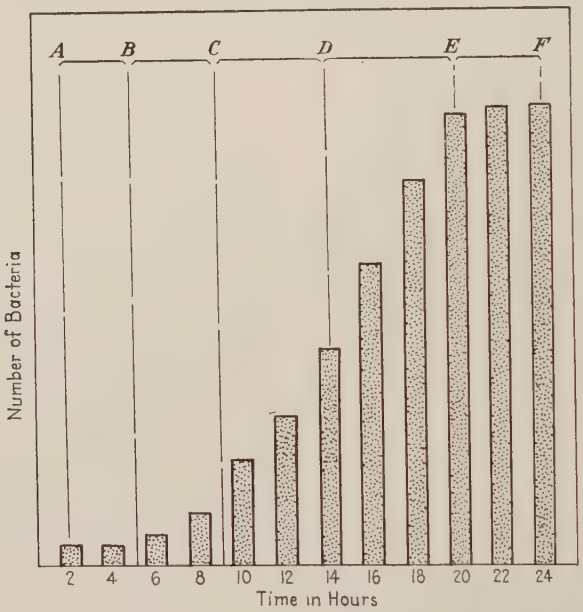


FIG. 80.—Increase in numbers in a bacterial culture indicated by height of the various columns; early lag period is followed by a rapid and accelerated growth and then a slowing down. (*After Buchanan.*)

period, in which the numbers remain constant; (4) *the period of decline*, during which the numbers diminish. Buchanan[4] still further subdivided the cycle (Fig. 80) into seven phases:

1. *Initial stationary.* No increase in numbers.

2. *Positive growth acceleration.* The organisms, meanwhile, are increasing in numbers and also increasing the rate at which they divide. This period is also known as the period of "lag."

3. *Logarithmic growth.* The minimum time occurs between generations, so that the rate of division is at a maximum through this period.

4. *Negative growth acceleration.* The rate of division is diminishing and the time between generations becoming longer.

5. *Stationary phase.* During this time the numbers are at a maximum, but the death rate is just the same as the rate of division so that no increase occurs in the numbers.

6. *Accelerated death rate.* The numbers which die are becoming greater than the numbers produced by division.

7. *Logarithmic death rate.* The deaths are increasing at a geometrical rate for which the divisions do not make compensation.

The first two of these periods are of interest since it would be expected that the divisions would start off immediately as soon as the organisms are free from the competition, which always occurs in old cultures, and have an abundance of food; but the curious phenomenon occurs, that, for a time, there is no growth. This phase is followed by a lag before the maximum number of divisions is attained. Chesney[8] has suggested that this condition, just after the transfer, is due to the presence, in the new environment, of some of the toxic materials which have been carried along with the bacteria. Clark and Ruehl[10] thought that, as these soluble deterrent materials diffuse out into the new media and the bacteria become freed from them, the rate of growth and division gradually speeds up until a maximum is attained. Soule[37] and Novy and Soule[33] believe a certain amount of CO_2 necessary in the cells for the best growth, and that this intracellular CO_2 is deficient in a very young culture.

Spores and Spore Formation.—Bacteria from an old agar culture show some of the rods no longer motile and with brilliantly refractive granules throughout the cell; in some, these will be seen to have gathered at the middle or toward one end (Fig. 78). These accumulations of granules fuse and develop a wall but do not swell the diameter of the cell which contains them. If such a culture is stained, the bodies of the bacteria take the stain, but the spores show as clear spaces in the cytoplasm. In *B. subtilis*, the spores are oval, 1.7 to 1.9 microns in length,[19] or, according to Prazmowski,[34] 1 to 2 microns in length by 0.6 micron in breadth. This difficulty of penetration by aqueous stains is due to the very thick wall impregnated with material of a fatty nature. If, however, a stain, dissolved in some substance which will mix with oil, is substituted, and is *boiled* into the spores, they stain so permanently that the dye can be removed only in acid alcohol. The walls of the bacilli forming spores do not hold the dye, so it is possible to bleach the bacteria and leave the spores still stained. The spore is usually stained red with fuchsin dissolved in 5 per cent carbolic acid, while the walls of the bacteria may be colored blue, methylene blue being ordinarily used; the red spores lie in the blue bacteria which formed them. The material from the bacteria has practically all passed into the spores so that the surrounding hull is simply the old bacterial wall and is nearly empty. The thick, fat-impregnated wall of the spore makes it very resistant to drying out or to the entrance of water or poisons into the living protoplasm in its interior.

The importance of the spores in bacteria was not recognized by Perty, who first observed them in 1852, and it remained for Cohn[11] to guess accurately as to their function in the continuance of the species when he noticed that cultures grown on solid media gave a deposit which was living, while the sediment in liquid cultures was dead, since bacteria do not form spores in liquids. He came to the conclusion that the brilliantly refractive little bodies which appear in *B. subtilis* rods grown on solid media were spores and of importance in the continuance of the species.

The varied results which had been obtained by the experimenters on the spontaneous origin of life when they boiled meat broth, the favorite culture medium, were due to the presence of these small reproductive bodies. The fundamental investigations of Brefeld[3] on their functions showed that a 20 minutes' boiling would not kill the spores, although an exposure to steam at 110°C. (under pressure) would, in 5 minutes. Gruber[20] inoculated a hay infusion with the spores, sealed the tube, and steamed it in a Koch steamer for ½ hour without preventing a growth appearing in a few days on the surface of each of the 6 tubes tried; 90 minutes' steaming had the same lack of effect. Blau[2] found that, while temperatures of 60 to 70°C. would kill vegetative forms (the ordinary growing organisms without spores) in 10 minutes,

the spores required a boiling of 175 to 180 minutes. Since the organism is widespread, it gets into and persists by means of its spores, in all sorts of food and media.

After staining by the method described nothing can be seen inside the spores themselves after they are completely formed. Nothing is visible which would serve to explain their great resistance to destruction by boiling or chemicals except the thickness of the surrounding wall.

Time Required for Spore Formation.—Migula[32] has found the time required for spore formation to be dependent on temperature; the more favorable the temperature is for growth, the more quickly the food is used in the culture media, and the sooner the spore formation begins, as illustrated by the following table:

At 14°C.....	72 hours required for spore formation.
18°C.....	54 hours required for spore formation.
20°C.....	48 hours required for spore formation.
25°C.....	40 hours required for spore formation.
30°C.....	38 hours required for spore formation.
35°C.....	33 hours required for spore formation optimum.
38°C.....	22 hours required for spore formation optimum.
40°C.....	38 hours required for spore formation.

It will be recalled that Brefeld found 30°C. produced the maximum growth, so that, if Migula is correct, higher temperatures hasten spore formation even if growth is not so large in quantity, *i.e.* growth and spore formation do not necessarily require the same optimum temperature.

Brefeld[3] did, however, claim that, in general, spore formation followed the temperature relations in growth, although the two were not exactly the same, spore formation being completed at 30°C. in 12 to 15 hours; at 25°C. in 24 hours; at 20°C. in 2 days; at 10°C. in many days; while at temperatures under 7°C. the spores were not observed at all. The cycle of development from spore to spore was completed in 25 to 30 hours at 30°C., while at 25°C., 2 days were necessary, and at 20°C., 4 to 5 days were required. The data furnished by those two observers, both of them experienced laboratory workers, do not at all agree, a fact that may be due to the type of organism with which each was working.

The rate of spore formation and of the cycle from spore to spore is of much practical importance in the sterilization of food material and of laboratory media. The usual practice to steam such substances every 24 hours for 3 successive days, if held at room temperature (18 to 23°C.), would prevent the formation of spores in the intervening intervals and kill those which had germinated.

Spore Germination.—Spore formation results in no increase in the number of individuals, since the spore gives rise to a single bacillus, which may later divide any number of times, but would start only a single colony. The spore imbibes water, swells, breaks open at the equator, and a new bacillus pushes out at the side of the spore.[34] This new organism is short at first but soon grows to the normal length of the *B. subtilis* although a very long time may be required for some of them. Acidity and alkalinity also affect germination.[22] The spores will not germinate at 5°C. in any hydrogen-ion concentration from 1 to 13, but at 25°C. they germinate within the limits 5 to 10, with the best germination at a pH of 7 to 8.

The Resistance of the Spores.—The spore is so different from the ordinary vegetative organism in its resistance to heat (wet or dry) and to chemicals, that some reason must be given for the change which spore formation has induced in its reactions to its surroundings. The explanation offered by Cramer,[13] that the protoplasm of the mold spores contains a carbohydrate which is usually insoluble but which may, at times, become soluble and increase the osmotic pressure in the spore cells has already been noted, page 107.

THEORIES TO EXPLAIN THE RESISTANCE OF SPORES TO EXTERNAL CONDITIONS

The bacterial spores, as compared to the ordinary mold conidia, have thick walls with special non-water-soluble impregnations, which make the loss of water from the inside very difficult, and the penetration of disinfectants, or of steam, from the outside very slow. Many of the early bacteriologists believed, as did Esmarch,[15] that the real power of resistance was in the thick walls which surrounded them. "The spores are surrounded by thick, dense walls which first must be softened before the heat can affect them."

Other authors had laid stress on the density of the protoplasm inside the spore wall as the source of the resistance. The evidence for this theory was summed up and much new data introduced by Lewith,[29] who called attention again to the fact that the proteins of the bacteria are coagulable just as are the proteins from any source and that death from heat is a coagulation process. Direct evidence is lacking, but the proteins of the bacteria can be brought into solution and then precipitated out, indicating that they are not different from other proteins. The coagulation of proteins depends upon the amount of water contained in them, as he showed in the following table:

Egg Albumin	Coagulation Temperature, Degrees Centigrade
In very watery solutions	56
With 25 per cent water	74 to 80
With 18 per cent water	80 to 90
With 6 per cent water	145
Dry albumin	160 to 170

The contents of the spores may not be albumin, although such a chemical would be indicated by their refraction of the light. Lewith estimated the contents of the spores to be similar to egg albumin which had a water content of approximately 10 per cent. The coagulation of water-soluble protein, therefore, is not alone a function of temperature and the time which the heat works, but also of the amount of water contained in the protein itself.

The drying out of the protoplasm by the extrusion of water as a spore is formed[39,21] in the molds and myxomycetes means an increased concentration of the materials in the protoplasm. The resistance of such spores to desiccation resulting from the removal of water and an increased osmotic pressure inside the protoplasm from the soluble materials would result; this increased osmotic pressure enables many of them to survive when they would otherwise perish, since the very thin walls found in many fungus spores would not preserve them from death by drying out.

Size of the Spore and the Vegetative Cell.—The material in the spore is very much more concentrated than in the vegetative cell, due to the extrusion of the superfluous water, and the spore is much smaller than the cell from which it arises. This condition is found in *B. subtilis*. With an average size of the vegetative cells of 2 microns in length by 0.375 micron in breadth,[1] the cubic contents of a bacterium is 0.22 cubic micron, while that of the spores ($0.5 \times 0.875\mu$) is 0.0734 cubic micron. The very thick spore wall is included in the latter figures, but even disregarding it, spores have a very much more concentrated material than do vegetative cells. The vegetative cells, however, have a much greater osmotic pressure than do the ordinary cells of the higher plants.

Another factor is the surface of the vegetative cell exposed. Using the same measurements as a basis, the surface of the bacillus cylinder is 2.36 square microns while that of the *outside* of the spore is 0.934 square micron. The lessened

external area of the spore would be diminished still further if the surface of the living protoplasm and not that of its thick wall is considered as the outside of the spore.

The possibility, too, must be suggested that the granules which seem to gather during the formation of a spore and which seem to be later dissolved inside the spore, becoming invisible, may have some influence on the osmotic pressure of the protoplasm, especially if they are carbohydrates, since carbohydrates develop much more osmotic pressure than do soluble proteins.

The resistance of the spore to external influences which would destroy the bit of living material not enclosed in thick walls is a fact, and a very important one so far as many of the commercial operations connected with food preservation are concerned, even if we have not arrived at a satisfactory explanation of its basis. The theory of Lewith seems to be the best offered, although other factors may be of importance.

The Plasmolysis of the Vegetative Cells.—If the cells of *B. subtilis* are placed in a 5 per cent solution of salt in water, it will be found that the protoplasm will pull away from the cell wall and a clear space, especially at the ends and in the middle, will show.[16] Even in a 2 per cent solution of salt some of the cells will be plasmolyzed. Fischer finds that 5 per cent is the usual strength in which bacteria are plasmolyzed, so that the hay bacillus may be regarded as typical.

The pressure inside the spores must be still higher but we seem to have no experimental data. The spore wall is not entirely impermeable to aqueous solutions, or the spores would never germinate when placed in liquid media, but penetration must be very slow. In disinfection with steam, flowing or under pressure, water must first find its way through the spore walls and soak up the very much dried protoplasm to reduce the osmotic pressure before the protoplasm will coagulate at any temperature used in sterilization; or, possibly, only enough moisture finds its way through the wall to start enzyme action which dissolves the wall in places.

The Spore Wall and Membrane.—The living membrane of the spore must play some rôle in the entrance of liquids into the protoplasm, since dyes, such as a weak Congo-red solution, will not enter living spores and the usual carbol-fuchsin solution will not stain the protoplasm, unless the spore has been previously killed by heat. Dead spores are penetrated by the dyes, and are stained a solid color, while the living ones show an unstained center surrounded by a colored ring.[7,24,30]

LOCOMOTION IN BACTERIA

The Organs of Locomotion.—These delicate little organs can be seen only if they are stained by special methods in which a heavy deposit around them is colored by one of the anilin dyes. After this treatment, delicate, thread-like projections (flagella or cilia) extend from all sides of the densely stained bacteria (Fig. 77); first photographed by Koch.[23] The attachment to the organism has never been studied in *B. subtilis;* whether it ends at the cell wall or continues through it to form a part of the living protoplasm is not known. Since the cell wall is not living substance and the flagella are the most active and living part of the organism, it would seem probable that they would be united with the inner cytoplasm and perhaps with the nucleus. The morphology of the flagella and their attachment have been studied in larger species where the details can be seen more easily and will be discussed later. Many interesting facts about the flagella of this organism are known, however, since it has been the subject of intensive investigations.[16]

Bacteria from old cultures, stained, show either none or only short flagella. In old cultures many of the bacteria are no longer motile, indicating that the presence of flagella is a sign of youth in cells and that as the cell ages, the flagella are lost.

The flagella may become stiff and useless even while they are still attached to the organism. The ability to bend easily seems to be characteristic of the young

specimens and they become brittle as the individual ages. If the hay bacillus is cultivated in a nutrient solution to which 4 to 6 per cent of ammonium chloride or 10 to 20 per cent of potassium nitrate is added, the organisms will remain motionless; if they are stained, the flagella will be found on them, as in normal specimens, but apparently still and inactive. At times, the flagella will be found to be all thrown off from the cells, lying free or rolled up in coils. The flagella seem to be especially susceptible to unfavorable conditions and are either lost or readily dropped off.

Cell Division and the Development of Flagella.—When a cell splits in two in the middle, the new end of each organism will be, for a time, without flagella, and those which covered the surface of the single short rod must now be spread over double the area. The flagella on the older organisms do not seem, however, to be any farther apart than those on the young ones, although the young ones must regenerate between them. Fischer could not find any short flagella interspersed between the

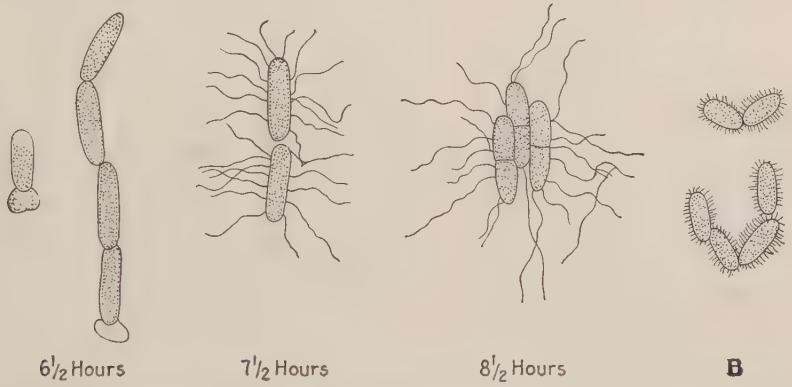


FIG. 81.—Development of flagella on *B. subtilis* cells arising from spores; very young cells 6½ hours after germination do not show them. *B*, the youngest flagella observed from another culture. (*After Fischer.*)

old long ones although they must certainly be there unless regeneration is entirely on the new ends. The formation of the new flagella on a new end is a very rapid process, requiring only 10 to 15 minutes.[32]

Fischer watched the development of the flagella on the young bacilli which come out of the spores of *B. subtilis* (Fig. 81). Three hours after placing them in a liquid culture at 30°C. he saw some swollen spores, many free bacteria, and a few chains with no movement visible. Even 6½ hours after the spores started to germinate, the motile bacteria were not numerous, although the organisms were very abundant. Some of the organisms, however, showed very fine short flagella over the whole surface. After 7½ hours, all of the bacilli seemed to be in active motion, and it was very difficult to find any that were stationary. Very short flagella were found on all the organisms. After 8¾ hours, the flagella had attained their full length, as was proved by examining them again after 22 hours. This growth time is much longer than that given by Migula, 10 to 15 minutes for a complete regeneration on a new end.[17]

Fischer was able in only a single case to see flagella on a spore-bearing organism. As soon as spore formation starts, the flagella become very brittle, so that in the staining operations all of them are lost and cannot longer be recognized. In a living condition, motile bacilli which contain spores can never be seen.

If the culture medium is made acid, the organism will still grow but not quite as favorably as in neutral media. The cells will be found to be in active motion, but if

stained, the flagella will be found to have been thrown off and to be lying around or in coils between them. Acidity seems to make them very brittle and easily lost.

The movement of the flagella cannot be seen in a living condition with the usual lighting, but it is possible to render them visible[35,6] by using the dark-field illumination. The movement of the flagella in *B. subtilis* must be very similar to that of a boat with a rudder and a row of paddles on either side. The side flagella propel the body forward and those at either end steer it. The flagella must be assumed to be, not only flexible but contractile. Their sides must be contractile or non-contractile at the will of the bacillus so that one side can contract while the other lengthens. The forward movement, therefore, would be due to the lashing of the water by the side flagella with the movements all coordinated and similar to that of whips.

The Rate of Progress Forward.—Although the motion seems to be quite rapid across the field of the microscope, it must be remembered that not only the organisms but also the path which they travel is magnified. Fried[18] found that at room temperature they could traverse 1 millimeter in 91 seconds, but that a higher temperature speeded them up so that they could cover the same distance in 43 seconds. This would mean a rate of 6 hours and 3 minutes for 1 inch, which is hardly express speed.

Looking at it from the standpoint of the size of the organisms, however, they are making rapid progress. Using Lehman and Fried's[28] measurements, *B. subtilis* travels three to four times its body length in 1 second at room temperature, the rate being 10 microns a second, or 1½ inches an hour. If a runner should make three to four times his body length (6 feet for example) in 1 second, he would run a mile in about 4½ minutes. These same investigators found another common organism, *B. vulgaris*, to have a speed of 14 microns per second, so that *B. subtilis* is not the fastest. Stigell[38] gives the average speed of the latter as only 3.58 microns per second, although it was the fastest bacillus whose motion was measured.

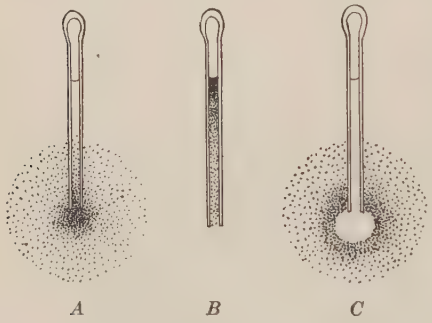


Fig. 82.—Capillary glass tubes filled with meat juice in drops of water containing bacteria: *A*, attraction to food substance diffusing from open end of tube; *B*, attraction to bubble of air in upper part of tube; *C*, repulsion in meat juice which has been made acid. (*After Pfeffer.*)

Reactions to Surroundings

Chemotropism.—*B. subtilis*, having organs of locomotion, is able to swim in search of new food or to avoid regions in which deleterious substances are in solution or food is scarce. The hyphæ-forming molds attain this object only by growth.

If capillary glass tubes filled with a nutrient substance (Fig. 82) are placed in water with a number of these organisms, the liquid will diffuse out of the open mouth of the tube into the water, they will become attracted to it, and divert their course to get into the more concentrated parts, finally swimming into the tube itself. This is *positive chemotropism.*

If the tube contains some injurious substance, such as a weak acid, the diffusion into the water will cause the motile forms to divert their course so as to avoid the more concentrated portions, and the mouth of the tube will be free from all bacteria, *negative chemotropism.*

Relation to Oxygen.—Some bacteria require oxygen and live only in regions where an abundance of it can be secured. If *B. subtilis* is stabbed into a tube of agar, the growth will not be along the entire line of puncture but confined to the surface of the liquid; it is an *ærobe*.

Engelmann[14] demonstrated the need of oxygen in some of the bacteria by placing them, along with some of the green algæ, in water on a glass slide. The green algæ produce oxygen and these oxygen-loving types collect in the regions along the algal filaments where the oxygen is being excreted (Fig. 83). Excluding the light stops oxygen production and the bacteria disperse evenly throughout the drop of water.

Relation to Carbon Dioxide.—In spite of their need of oxygen, a certain amount of carbon dioxide must also be present or *B. subtilis* will not grow.[37] The CO_2 is

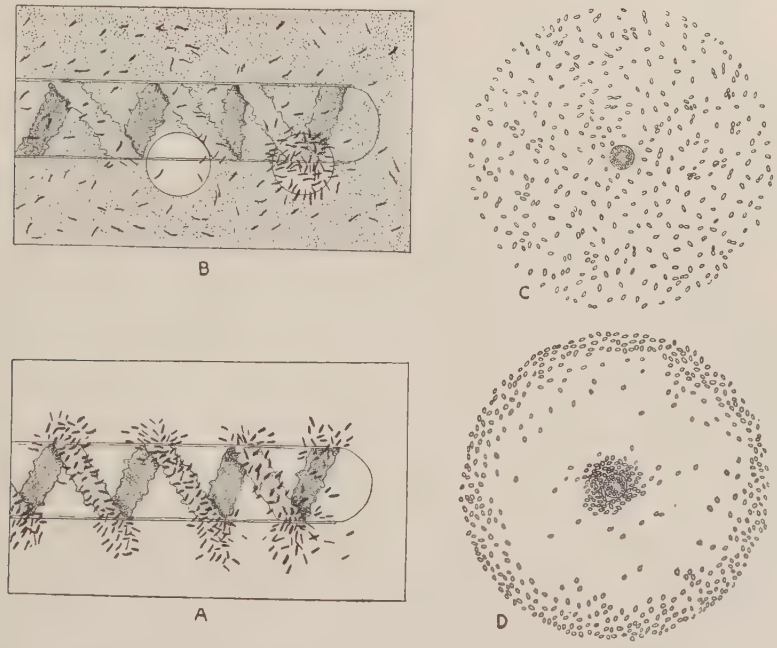


FIG. 83.—Effect of excretion of oxygen on aerobic bacteria: *A*, bacteria clustering around chlorophast of Spirogyra filament; *B*, field of microscope with two openings, one *opposite* turn in chloroplast, other *between* turns; *C*, one-celled alga, dark field; *D*, same illuminated, bacteria within influence of excreted oxygen gather around cell; bacteria on margin are too far away. (*After Engelmann.*)

held in the body of the organisms as an *intracellular* gas, and if this small amount is removed, growth is stopped.

The Effect Which B. Subtilis Has on Various Culture Media.—*B. subtilis*, inoculated into a tube of gelatin, will grow largely on the surface of the gelatin to form a crater of liquefied gelatin in the center of the tube. This liquefaction is the result of the activity of one of the enzymes which digest gelatin, the *proteoclastic type*, as can be demonstrated by adding a disinfectant to the liquefied portion and pouring it into a fresh tube of gelatin, which will, in turn, liquefy.

Inoculated into a tube of sterile milk, *B. subtilis* will coagulate the milk with one of the *rennet enzymes*, but the clotted milk can be broken up very easily. If

allowed to remain for some time in the milk, it will gradually digest this curd, so it is evidently provided, also, with *casein-dissolving enzymes.*

B. subtilis in sugar broths will grow well, but will not produce gas by the destruction of the sugars, so that it is not classed as one of the gas producers. It will, however, produce acid.

If inoculated into blood serum, it will grow but will not liquefy it, so that it is not provided with a serum-dissolving enzyme.

SUMMARY

A Technical Description of the Organism.—From the facts learned above and from other similar laboratory tests, the bacteriologist compiles his description of this organism. These descriptions are based upon the *morphology of the organism* as it appears in cultivation on the ordinary laboratory media, including its reactions to some of the stains which have been used extensively, one of which is the Gram stain; many organisms have the same size, proportions, grouping, and staining reactions so that this record must be amplified by a complete account of its *physiological and biological reactions* to the various media. This description may be illustrated for *B. subtilis* by the account based on Soule's recent[37] work:—

Bacillus subtilis (Ehrenberg) Cohn (Ehrenberg, Infusions thierchen als volkommene organismen, Leipzig, 1822; Cohn, Beiträge z. Biol. 1, 1875).

Form.—Cells occur simply or in pairs with rounded edges; usually about 0.8μ by 3 to 4μ in length; filaments may be present.

Stains.—Stains readily. Gram positive.

Motility.—Single cells and chains are extremely motile with a characteristic snake-like movement. Cells lose their motility with age.

Sporulation.—Large oval spores near the middle without enlargement of the cell.

Broth.—Diffuse growth for 3 to 4 hours; in 18 to 24 hours granular precipitate on side and bottom of tube with clearing; after 24 to 36 hours, a tough pellicle is formed from union of the isolated pellicles, which, if undisturbed, will remain on surface of tube.

Gelatin Colonies.—Liquefies gelatin rapidly. Colonies on gelatin have a grayish central mass surrounded by a lighter granular zone. Edge of colonies consists of dense zone of bacilli arranged radially to form a "ray crown."

Gelatin Stab.—Liquefaction along entire line of inoculation forming a saccate growth. Flocculent masses accumulate at the bottom of the liquefaction crater.

Agar Colonies.—Whitish, slightly glistening, moist, soft, smooth, compact, regular and fluorescent, when young. Opaque and markedly granular when old. Friable. Center is dense and no "ray crown" appears. Colony composed of short bacilli.

Potato.—Smooth, thick, moist growth with velvety appearance.

Similar descriptions are compiled in the form of manuals, such as Bergey's Manual of Determinative Bacteriology,[1] for all the known and described organisms with sufficiently well defined characters to make them possible species. The bacteriologist, after growing an unknown organism in the laboratory on various media, can compare its morphology and reactions with those already known and decide as to its name and relationships. The task is more difficult, it will be seen, than that of the zoologist or botanist who uses his manual to determine the name or relationships of a newly found plant or animal, since not only the morphological characters must be determined, but also the physiological ones, and the latter are often more difficult. The method of combining physiology and morphology to determine the relationships of an organism is the best that has been evolved and will probably continue to be used until a better one is found.

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CHAPTER XX

FOUR OTHER COMMON BACTERIA

BACILLUS MESENTERICUS VULGATUS (THE POTATO BACILLUS)

(*Bacillus vulgatus or Bacillus mesentericus*)

If an ordinary potato, after being peeled and washed, is placed in a covered jelly glass or tumbler and steamed for ½ hour, there will appear, after an interval of 5 or 6 days, dirty white colonies on its surface. After a few days of growth, these colonies will develop a skin, *pellicle*, which, becoming very convoluted and wrinkled, resembles a mesentery. This organism is probably *Bacillus mesentericus vulgatus*, according to the older name, or *B. vulgatus* or *B. mesentericus*, according to the more recent manuals. Gottheil's *Bacillus graveolens* is also probably the same organism.

The name indicates its resemblance to a mesentery and that it is widespread, *vulgatus*. As it is one of the very common soil organisms,

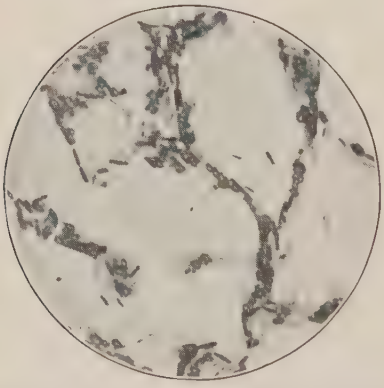

FIG. 84.—*Bacillus vulgatus* without spores. (*From Kral Institute.*)

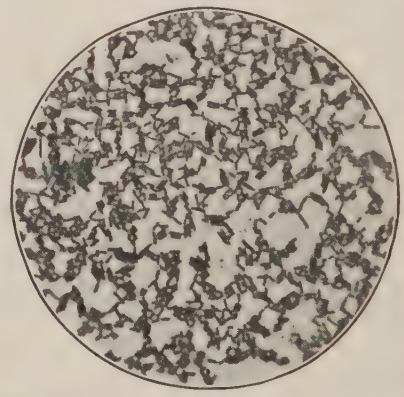

FIG. 85.—*Bacillus vulgatus* with spores. (*From Kral Institute.*)

it is always found on the skin of potatoes, from which no ordinary washing will remove it. Under certain conditions, such as high temperature and humidity, some strains of this organism will rot potato tubers in storage.[5]

The Organism.—The bacillus in ordinary meat extract media is a short rod about 1.5 microns in breadth and 3 microns long (Figs. 84 and 86). In old cultures, the cells become filled with fat droplets.

The spores, about 1.5 by 2.5 microns, are formed in about 36 to 40 hours on slices of potato or other solid media, the spores being placed about the middle of the bacilli, as in *B. subtilis*, but causing the organisms enclosing them to swell somewhat. According to Gottheil[18] the spores are a little smaller than in the hay organisms. They germinate from the side in about 5 to 6 hours at 28°C. and a bacillus pushes out as in *B. subtilis;* germination in the latter organism is occasionally from the end.

The spores are even more difficult to kill than those of the hay bacillus and, as they are very abundant in the soil, any substance contaminated by soil, or soil itself, is difficult to sterilize. The importance of the organism lies in the resistance of the spores to heat, as first noted by Globig,[17] who isolated a series of organisms from the soil by growing them on blood serum. Many of them were undoubtedly the potato bacillus since, of the 30 cultures which he obtained, all but 2 were rod-shaped forms producing spores.

Slime Production.—An examination of a culture of this organism growing on the potato shows that the wrinkled growth is held together by slime, and a transfer on

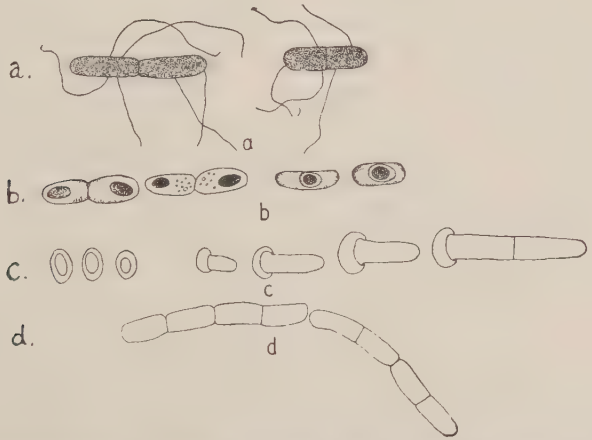

FIG. 86.—*Bacillus vulgatus: a*, typical flagellate forms; *b*, spore formation; *c*, spore germination; *d*, chain of organisms arising from the germination of a spore. (*After Gottheil.*)

a platinum needle will draw out into a long slimy string. The organism is a slime producer with each cell enclosed in a layer of mucilaginous material. Slime production is of importance in a related organism, *B. panis*, which causes slimy or ropy bread.

Occurrence in Milk.—Milk always contains numbers of this organism derived from the soil, and its presence renders sterilization of milk very difficult, owing to the heat resistance of the spores. Strub[40] cultivated this organism, isolated from milk, on pieces of potato and then dried them on bits of silk thread. Spore-bearing silk threads were subjected to streaming steam for varying lengths of time, from 20 minutes to 1½ hours, the latter time being necessary in most cases to destroy the vitality of the spores. Even an intermittent steaming for 3 days was not sufficient to kill the spores. Wroblewski[44] found that tubes of broth inoculated with this organism required 3 days' heating to a temperature of at least 80°C. for 20 minutes, with 12-hour intervals, in order to kill the spores. From his trials made with milk at 70°C., it is evident that if many *B. mesentericus vulgatus* are present, it will still contain a large percentage of viable spores after pasteurization.

BACILLUS MYCOIDES (THE FUNGUS-LIKE BACILLUS)

Some of the commonest colonies which will appear on agar plates exposed to the dust of the air, or on plates containing soil dilutions, will be large, white, thread-like growths looking very much like fungi and for which they easily may be mistaken. Because of their resem-

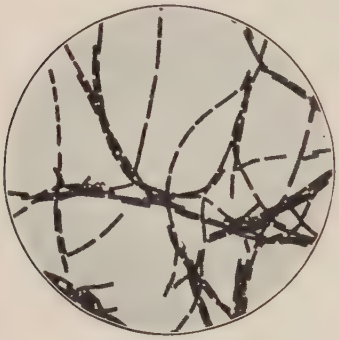

FIG. 87.—*Bacillus mycoides*, without spores. (*From Kral Institute.*)

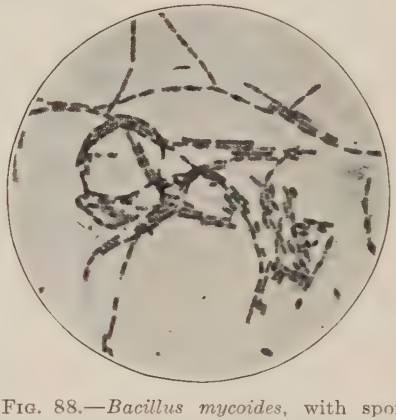

FIG. 88.—*Bacillus mycoides*, with spores (*From Kral Institute.*)

blance to the fabled Medusa with the serpents in her hair, these colonies are called, "medusoid." Such a colony, under the microscope, shows long strands composed of parallel chains of rod-shaped bacteria extending from it (Fig. 87).

FIG. 89.—Germination of spores of *B. mycoides: A*, the three forms of germination of the spores in a strain showing polar germination with one germ tube, *a*; equatorial germination with two filaments, *b*; and polar germination at both ends, *c*; another strain is shown in *B* with a single filament coming out of the spore at *d* and two filaments at *e*. (*After Holzmüller.*)

The Organism.—The rods are a little longer than those of *B. vulgatus*, averaging 1.3 by 3.5 microns (Fig. 88). They are motile by flagella when young, but they settle down, the organisms lose their flagella, divide, and form long chains with a spore, about 1.8 by 2 microns in size, at the center of each cell.

The Colonies.—The most striking feature about this organism is the thread-like rows of cells, readily traced at the margins of the colony, extending out, side by side, like a series of parallel railway trains. The chain (Fig. 91) becomes double at a bend,

both sides of the bend continuing as a row of cells. Such a doubling may be formed at any point where a sharp bend occurs.[18,19] Pringsheim and Lauger[33] have found that the threads have a marked tendency to turn to the left, for some unknown reason, but the peculiar shape of the colonies is characteristic and is not the result of lack of food. A high concentration of the materials in the agar, however, will cause a stronger bending of the threads.

Perlberger[32] claims that *B. mycoides* loses its ability to produce mycelium-like colonies after it has been cultivated for years in the laboratory, although a few strains did retain this character. The size of the cells did not vary in spite of years of cultivation.

The Spore Germination.—The spores (Fig. 89) are similar to those of *B. subtilis* and *B. vulgatus* but germinate at the ends (polar) and not at the middle (equatorial).

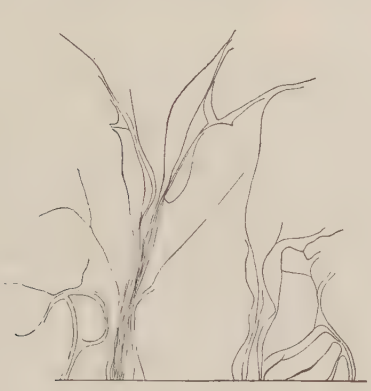

FIG. 90.

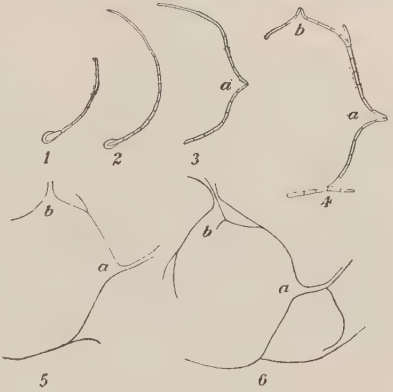

FIG. 91.

FIG. 90.—Edge of colony of *B. mycoides*. (*After Holzmüller.*)

FIG. 91.—Formation of colony of *B. mycoides:* 1 and 2, germination of spore and beginning of formation of a filament; 3, filament bending sharply at *a* begins to break into two segments; 4, another break at *b* and two filaments at *a*, growing out parallel; 5 and 6, further branching and breaking of filaments to form parallel filaments. (*After Holzmüller.*)

Instead of germinating at the poles, however, a cell may continue to grow (Fig. 89), held inside the spore by the ends, until it bursts the wall at the equator and two bacilli emerge. On the other hand, both ends of the spore may give way and a chain of bacilli extend through the old spore case. Like *B. mesentericus vulgatus*, this bacillus stores fat in the form of droplets in the cells.

Time Required for Spore Germination and Formation.—About 17 to 18 hours are necessary for the spores to germinate and return to the spore condition in nutrient media, as the following life cycle[28] shows:

12:30 p.m.	Spores only.
1:30 to 3:30	Vegetative growth, some spores.
4:30 p.m. to 1:30 a.m.	Vegetative growth only.
2:30 a.m.	Vegetative cells show internal granulations.
3:30 a.m.	Vegetative cells show marked granulations.
5:30 a.m.	Granules unite in cells.
6:30 a.m.	Complete spores.
7:30 a.m.	Abundant spores.

Physiology of the Spores.—The organism is widespread in the soil and finds its way into dust, water, milk, and food. The spores are very resistant to desiccation,

steam, heat, and sunlight. Holzmüller[19] tried to kill the spores by exposing them to diffuse daylight but found it to be entirely without effect. He then resorted to exposing them to direct sunlight in Petri dishes on agar, from 7:30 a.m. until sundown, but they germinated almost as usual after 48 hours' incubation; 8 days' exposure was necessary in order to make any impression and, even then, they were only injured (not killed) as indicated by the number of involution (abnormal) forms. The organism formed spores even in the direct sunlight, so that on these bacteria sunlight would not act as a germicide, although usually it is an excellent and cheap one.

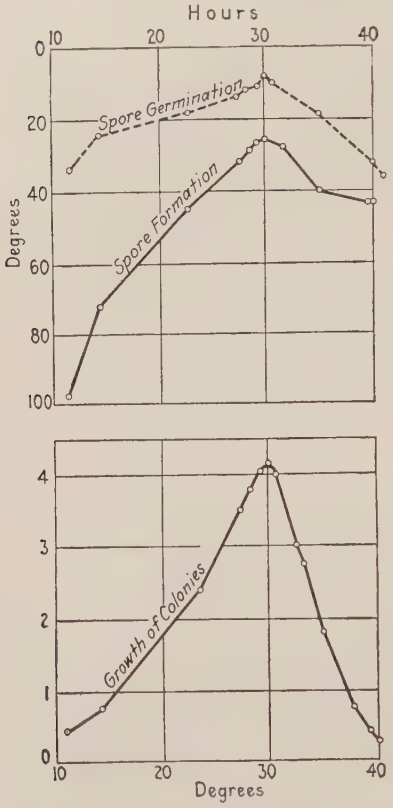

FIG. 92.—*B. mycoides;* spore formation, spore germination, and growth of the colonies at various temperatures. (*After Holzmüller.*)

The same author investigated the effect of *temperature* on some of the life processes in an attempt to discover optimums for: (1) *growth,* as measured by the size of the colonies; (2) *spore germination,* as indicated by the length of time required in minutes; and (3) *spore formation,* in minutes required. The interesting fact appears, from the curves in Fig. 92, that the optimum temperature is about the same for all three of these life processes. The particular type of protoplasm which makes up *B. mycoides* works best at about a certain temperature regardless of the phase of life history.

The thermal resistance of the spores is largely dependent upon the environmental conditions under which they were formed and have been kept.[28] Their age, together with the temperature and humidity of the environment, determines their heat resistance.

By selecting those which have escaped destruction by heat, a strain of *B. mycoides* may be developed whose spores have a thermal resistance at least twenty-five times that of the original strain.[28]

BACILLUS FLUORESCENS LIQUEFACIENS (THE FLUORESCENT BACILLUS)

(*Pseudomonas fluorescens* (*Flugge*) *Migula*)

Plates made from sewage contain large white colonies, with a green, or greenish yellow, transparent, coloring matter diffused in the medium around them. This coloring matter changes from a green to a greenish yellow, or, if the dish is held to the light, and the media looked *through* and not *at,* will have a yellow tint, a property called *fluorescence.* The physical explanation for this phenomenon is not clear.[1]

Similar organisms occasionally may be obtained from plates made from milk, soil, or water. One of their commonest locations, in

the maple sugar region, is in the pails of maple sap collected at the trees in the spring when the sap is flowing. The organism becomes very abundant in the pails after they have hung for some time on the trees and have become thoroughly contaminated from rain water flowing down over the bark of the trees into them. The latter part of the maple sap season is usually rather warm, and the organism has a chance to grow in the sweet juice, which furnishes all the necessary nutritive substances, both mineral salts and carbon-nitrogen compounds.[13]

Edson[11] found fluorescent bacteria playing a very important part in the deterioration of maple sap in the sap buckets. The early "runs" of sap, as clear as water and with a clean sweet taste, change with the coming of the warmer weather, become more or less cloudy, and develop a disagreeable flavor; such sap is said to be "sour." Occasionally it is green in color, but in all cases the fluorescent organisms are the prevailing type.

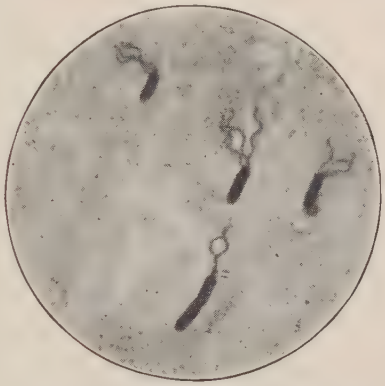

FIG. 93.—*Bacillus fluorescens* from maple sap; stained to show flagella. (*From Edson.*)

The Organism.—The organism is a rod, with about the shape of a coffee bean, with rounded ends, occurring singly, or in pairs, and, occasionally, in chains composed of 5 to 7 individuals. The length is from 1.5 to 3 microns and the width is about 1 micron, or slightly less. The organism will grow to a length of about 3 microns and then split into two individuals each 1.5 microns. They are all actively motile in young cultures and stained preparations indicate that the flagella are from 1 to 6 in number and are all attached at the ends of the organism (Fig. 93). Such a type of organism, with polar attachment of the flagella, was called a *Pseudomonas* by Migula.[31] Burckhardt[6] believes that the number of flagella is of greater importance and more reliable as a distinguishing character for species than biological characters, such as liquefaction of gelatin or the production of pigments in the media.

The Action of the Flagella.—The flagella on the sides of *B. subtilis* act more in the nature of oars or as whips whose sharp contractions drive the bacillus forward, while those at the end serve as rudders to steer the individual and, perhaps, as propellers. In an organism such as *B. fluorescens*, the polar flagella must both propel and steer.

Reichert[34] studied the movements on one of the corkscrew-shaped types, *Spirillum volutans*. The organism has a single flagellum, making the movements easy to follow. Reichert confirms the theory of Bütschli[7] and Delage and Herouard[10] that the flagellum acts as a screw pushing the organism forward in the same manner that the propeller on a steamer bores a hole through the water. The flagellum rotates around its axis; the attachment is at the posterior end of the organism and pushes the body of the organism ahead, as is shown in the diagrams illustrating the movements of force (Fig. 94*a*). The flagellum, in all the species studied by Reichert, swung in the form of a right-handed screw, due to contraction waves which traverse its length. The rota-

tion of the flagellum to the right swings the organism to the left (Fig. 94*a*), the flagellum stroke swinging the organism first to one side and then to the other. The movement of the flagellum itself has been compared in shape to a funnel. Metzner[30] ascribes great importance to this conical vibration and believes that, by means of it, the organism sucks its way through the liquid. The flagellum can act on the front end of the body just as well as on the posterior, and sometimes it is found at one end, and sometimes, at the other, but in most species, especially the bacilli, the movement is from

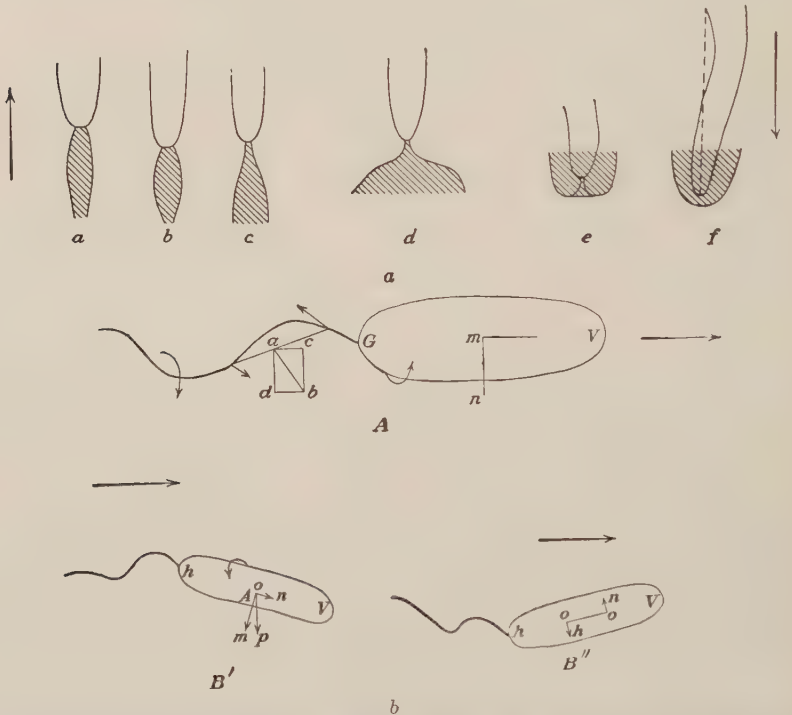

Fig. 94*a*.—The polar flagella of *Thiospirillum jenense* swing through the shaded area: *a* to *d* are attached at the posterior end; *e* and *f* at the anterior end. The forward movement of a single polar-flagellated organism is complicated by a funnel-like movement which results from the swing which the flagellum gives the body of the bacillus. 94*b*. The components of the force *ab* exerted by the flagellum brings the body into a new position each moment that the force is exerted. The same effect is shown in *B′* and *B″* where the flagellum swings any particle in the body from a straight line and forces it to assume a series of positions which give the bacillus a swaying movement. (*After Buder and Reichert.*)

the rear. While the organism is in a resting or quiescent condition, the flagellum is extended to some length, but in quick motion, it is shortened.

The general facts about polar flagella apply as well to flagella on all parts of the body, in so far as their contraction and whirling are concerned. The theoretical explanation of the contraction and expansion of an apparently homogeneous structure is very difficult, and Metzner[30] denies that they are homogeneous in Spirillum, claiming that they are made up of a number of strands which can be separated into individual flagella, that is, the single flagellum is really a compound structure. A similar structure of the flagellum may occur in the sulphur bacteria.[25]

The Fluorescent Green Pigment.—The production of the fluorescent, green-yellow pigment and the conditions under which it is produced have attracted much attention. Jordan[22] investigated the effect of various mineral salts and a variety of carbon and nitrogen compounds upon the amount of color produced in *B. fluorescens liquefaciens* and a number of related forms. Both phosphorus and sulphur seem to be necessary for its production, but the nature of the phosphorus or sulphur compound makes little difference. The calcium compounds may be omitted without any observable effect on color production, but magnesium is absolutely essential. Thum[41] has already observed this same fact and it confirmed the work of Kossowicz[27] on color production in the pink yeasts. Edson[11] observed that strains of the fluorescent organism which grew vigorously were, as a rule, strong color producers also. This observation is in agreement with Jordan's theory that color production is of no advantage to the organism, but is simply the expression of certain changes wrought by the organism upon its substratum. With certain compounds in the substratum, the metabolic activities of the organism adjust themselves to these conditions, and the excretion products correspond. It is purely a matter of accident and of no physiological significance that, under certain conditions, one of these metabolic products happens to be fluorescent; active growth means a rapid change in the substratum, and a fluorescent color production is one of its phases.

Bacillus Pyocyaneus (Pseudomonas aeruginosa (Schröter) Migula).—This related species occurs in wounds and on surgical dressings where antiseptic precautions have not been observed, infectious gangrene being a regular occurrence in surgical operations in the days before contaminations were avoided by sanitary precautions. The organism occurring in water, sewage, etc., is known as *Pseudomonas fluorescens liquefaciens;* that infecting surgical dressings as *Ps. pyocyaneus* (*Ps. æruginosa*). The former organism also appears as a distinct species or strain which does not liquefy gelatin, and is known as *Ps. fluorescens.*

A comparative study of *Ps. fluorescens* and *Ps. pyocyaneus* convinced Ruzicka,[36] cultivating a number of strains in the laboratory, that the two were distinct species, the principal difference being in the optimum temperatures required; *Ps. fluorescens liquefaciens* died off in animals, although it produced an inflammation; while in water, *Ps. pyocyaneus* disappeared long before the other organism had been killed. But, Ruzicka[36a] in a later paper is not sure that the two are distinct, as the characteristics of the two organisms are very variable. Strains of *Ps. fluorescens liquefaciens* grown first in the warm incubator and then in the bodies of guinea pigs were very similar to the other type. *Ps. pyocyaneus* could be adapted gradually to saprophytic conditions of life in water, a stream of air through the culture helping in the alteration. The acquired characters were again soon lost in cultures, but on the agar slant the changes in *B. pyocyaneus* were permanent for almost a year before it reverted.

Although *Ps. pyocyaneus* alone can bring about pathological conditions, it is usually found associated with streptococci and staphylococci. It probably serves to utilize some of the products of the poisoned body cells by the proteoclastic enzymes which it secretes. These enzymes of the fluorescent organism, however, do not seem to dissolve the other bacteria with which it associates, although in liquid cultures there is some digestion of the tuberculosis organism if grown with it.[26]

Color Production in B. Pyocyaneus.—Gessard[14] discovered the importance of phosphorus in the formation of the blue-green pigment experimentally; with the addition of only 0.125 grams of potassium phosphate to 1 liter of culture medium, the green color would appear but fluorescence be lacking; if the phosphate were increased to 0.130 gram to the liter, fluorescence was noticeable. The fluorescence may be of biological importance to the organism, but he was unable to point out where it would be essential.

The fluorescent green-yellow color of the ordinary strains of the saprophytic organism of the *Ps. fluorescens liquefaciens* type is accompanied, in *Ps. pyocyaneus*, by a second pigment of a deep blue tint which gives it a much darker green color.[22] The second pigment can be extracted from the aqueous solutions by the addition of ether, is not poisonous to animals, and is not the source of the sepsis which, in wounds, marks the presence of this organism. The suppurations which follow the entrance of *B. pyocyaneus* into wounds is probably the result of (1) a toxin which poisons the cells, (2) its marked proteoclastic properties.[23]

In a medium composed of mineral salts, ammonium phosphate, and 6 per cent olive oil, the bacilli produce a rose-red coloring matter, but if the medium contains the amino acids, alanine, leucine, or asparagin, as a source of nitrogen, instead of ammonium phosphate, a blue-green pigment, apparently *pyocyanin*, is formed. The coloring matter results from the destruction of some of these amino acids, but it cannot be built up from an organic salt, such as ammonium phosphate. With urea as a source of nitrogen, along with one of the sugars, such as glucose, maltose, lactose, or galactose, the color production is only slight and a yellow or green.[38]

Pyocyanin may be extracted from cultures by adding chloroform and alkali, its dark-red salts being much easier to obtain pure than the coloring matter itself whose dark-blue crystals are very unstable.[29] Its approximate formula seems to be

$$C_{26}H_{25}O_3N_4$$

It is soluble in hot water, dilute alcohol, chloroform, acetone, or ethyl acetate, but is insoluble in ether, benzene, xylene, carbon tetrachloride, light petroleum, or carbon disulphide. It is strongly basic.

The color production in most of the fluorescent organisms can be stopped by growing them at an unusually high temperature for a few weeks. If *B. pyocyaneus* is incubated for four generations at 42°C., the succeeding generations will be colorless[9] and repeated inoculation under the skin of frogs will not restore color production. If the color is still being formed, the amount and intensity can be increased by such inoculations, but, with no color, the production cannot be revived. Color production in other bacteria also (if it once has been lost by growing them at a too high temperature) is very difficult to start anew, although Rochaix and Bausillon[35] claim to be able to revive it by growing *B. pyocyaneus* in a 15 per cent solution of glycerine, levulose, or glucose in peptone water.

Action of B. Fluorescens on Proteins and Sugars.—The ability to split proteins is very strong in *Ps. fluorescens*, and in the soil it is one of the most abundant organisms and, probably, one of the most active protein destroyers.[39] Emmerling and Reiser[12] tried its effect on gelatin tubes and observed that, while the gelatin liquefied at the top, it remained solid at the bottom, the gelatin being ammonified at the rate of about 25 per cent a month under laboratory conditions. None of the intermediate products, such as phenol, indol, skatol, or hydrogen sulphide were produced, but some amino acids, such as methylamine and trimethylamine, choline, and betaine, were obtainable in sufficient quantities to test. It is not a producer of poisonous ptomaines but peptonizes proteins and converts them into amino acids. If grown on blood fibrin, tyrosine, arginine, leucine, or aspartine are the products of its activity, indicating a slow enzyme. Simpler nitrogenous compounds, such as urea, are broken into ammonium compounds and sugars, such as saccharose, maltose, and lactose, are split.[37] The odor produced by *Ps. pyocyaneus* is the result of the splitting of tryptophan and will not appear unless this amino acid is in the medium.[15] Edson[11] was able to destroy urea with any of 13 strains of fluorescent organisms, except *Ps. mesenterica*. They all grew well on peptone and asparagin, but on ammonium lactate, 2 strains refused to grow; while on the inorganic sources of nitrogen the results were still poorer, only 3 strains growing on ammonium phosphate, and on ammonium

tartrate only 7. All but 2 of the strains grew well on Uschinsky's solution with the carbon and nitrogen as glycerine, ammonium lactate, and sodium asparaginate. All of these organisms, except one, produced alkali on lactose-peptone broth during the first few days of growth but after 20 days, 8 of the 20 were producing acid. On dextrose peptone, nearly all produced acid, while above half of those isolated from maple sap produced acid and the other half produced alkali on sucrose peptone. The fluorescent bacteria in the maple sap buckets in the spring cause a deterioration in the syrup produced, both in its color and flavor. The sugars in maple sap form about a 3 to 5 per cent solution of which 95 per cent is saccharose. The fluorescent organism acts on these sugars, probably inverts part of the cane sugar, and converts it into acid, especially during the latter part of the season.

Four per cent saccharose in broth cultures of *Ps. fluorescens*, if kept slightly acid, will invert; but if alkaline, the inverting enzyme will not function. Sugar solutions protect this enzyme from high temperature, while in water (in which it is very soluble), it is very easily destroyed.

Bios and the Growth of Ps. Fluorescens.—As noted under Yeasts, certain organic substances seem to stimulate growth in a manner not duplicated by inorganic salts. The bacteria have been also the subject of investigation by students of "bios"; Izrailski and Runov[20] obtained from the juice of the potato tuber passed through a Pasteur-Chamberlain filter a substance which would stimulate the growth of *Ps. fluorescens*. This compound could not be precipitated by the addition of alcohol but was absorbed to some extent by the kaolin of a filter. These stimulating substances have been proved in the yeasts not to be vitamins (p. 140).

BACILLUS PROTEUS VULGARIS (ORGANISM OF DECAYING PROTEINS)

In meat kept for some time, a very variable, rod-shaped organism develops. The organism can be separated readily from the other types found in putrefying meat by plating out with gelatin instead of agar. The organism is a rapid and profuse grower, of the type called a "spreader," and liquefies gelatin.

Cantu[8] was able to isolate *B. proteus* from any putrefying meat and also from spoiled eggs and fish. His method was to pick it up pure in nearly all cases from the spreading amoeboid colonies on the surface water on top of the gelatin (or agar) plates. Wenner and Rettger[43] have somewhat improved his methods. The organism is one of the easiest to grow in the laboratory, flourishing luxuriantly on any of the ordinary types of meat media, at temperatures from 20 to 37°C.

The name, *Proteus*, is given because of the numerous forms which it assumes; Proteus, the Homeric Greek "Old Man of the Sea," who tended the flocks of Poseidon, had the gift of transforming himself into an endless variety of shapes.

The Occurrence of the Organism.—The organism is very common not only in putrefying meat, as already noted, but also in garden soil from which Cantu[8] was able to isolate it from 23 out of 52 samples which he tried, as well as from spoiled meat, eggs, and fish.

It is rarely pathogenic and then usually only in association with some other organism, although it does occasionally produce infection in war wounds.

The Organism.—The rod-shaped organisms vary in size from 0.5 to 1.0 micron in breadth by 1.0 to 3.0 microns in length and appear singly, in pairs, or in chains. They are motile by flagella attached to all parts of the body (Fig. 95). The mor-

phology is so variable that other characteristics are studied. Its outstanding physiological property is the ability to digest proteins of many types to form alkaline products. This bacillus is important in nature, serving as one of the active ammonifying agents in the soil, breaking proteins, especially those of animal origin, and forming hydrogen sulphide in abundance.[2] The free amino acids in the protein medium seem to determine the avidity with which the organisms destroy it.[3]

While the liquefaction of gelatin and the destruction of proteins, due to an enzyme resembling trypsin,[24] is considered to be the outstanding feature of the group, Wenner and Rettger[43] found even this property so variable that they attempted to separate species and strains on the fermentation of sugars, such as sucrose and maltose; one group ferments maltose and the other is unable to attack this disaccharic sugar, the former group being the original *Proteus vulgaris* of Hauser. *B. proteus* belongs to the *colon-typhoid* group of bacteria which has been monographed by Weldin.[42]

In fact, liquefaction and digestion of gelatin may be only a secondary character, since the organisms of this group apparently more easily attack carbohydrates than they do nitrogenous substances.[16] They liquefy gelatin much more easily when no sugar is present than if any of the sugars are supplied. The addition of the sugar also inhibits the formation of *indol*, since the conversion of sugar into some of the acids,

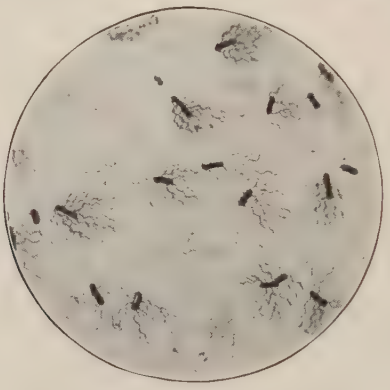

Fig. 95.—*Bacillus proteus vulgaris*, with flagella stained. (*From Kral Institute.*)

such as lactic acid, makes the medium too acid and growth ceases; carbohydrates are always used in preference to proteins.[4,21]

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CHAPTER XXI

BACILLUS COLI

(THE COLON BACILLUS)

[*Escherichia coli* (*Escherich*) *Castellani and Chalmers, Escherichia communior Durham*]

The so-called "colon bacillus" is one of the commonest normal inhabitants of the intestine of all warm-blooded animals, passing from the intestine into soil and water where it remains for some time in a living condition. It may possibly adapt itself to the colder surroundings of the soil or water, and strains of the organism which are able to continue under the new conditions may survive. Escherich isolated it first from the feces of a cholera patient in 1884, suspecting it to be an active pathogene producing the disease, but it was soon shown to be a normal and apparently harmless inhabitant of the undiseased human intestine and easy to isolate from contaminated soil or water.

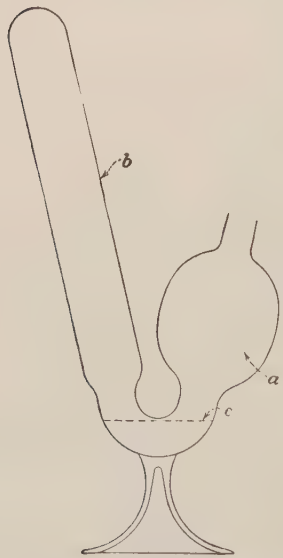


FIG. 96.—Fermentation tube devised by Theobald Smith. The gas is collected in the closed arm *b*.

B. coli is usually considered to be entirely saprophytic, but will produce disease if it gets into the proper conditions having been found in cases of appendicitis. In constipation, quantities of it sometimes occur in the urine, having apparently penetrated the walls of the intestine and been absorbed into the blood stream, to find its way through the kidneys into the urine.[37]

Isolating the Organism.—The easiest method to obtain it in pure culture is from water contaminated with sewage (Standard Methods, Water Analysis).[35] A cubic centimeter of such water is placed in a fermentation tube (Fig. 96) filled with beef broth containing 1 per cent of lactose and the tube is incubated 24 hours at a temperature of 37°C. If the tube shows, at the end of that time, an abundant growth of bacteria in the open arm, and if the closed arm is from one-fifth to one-half filled with gas, some of the material is diluted heavily with sterile water and plated out on lactose agar with litmus solution to give a blue color. Some of the acid-producing colonies inoculated into a fermentation tube will be found to be active gas producers, 20 to 70 per cent of the closed arm. As a further test, similar fermentation tubes to which an anilin color, *neutral red*, has been added in sufficient quantity to give the broth a cherry-red color, may be inoculated. After incubation at 37°C. for 2 days, if the organism is *B. coli*, the gas will be produced as before, but the bright-red color

of the broth in the closed arm will change to a brilliant canary-yellow. The gas constituents may be determined by marking the amount on the closed arm, filling the closed arm of the tube with a 5 per cent NaOH or KOH solution, washing the gas from the closed arm, back and forth in it, and returning it, finally, to the closed arm, the mouth of the open arm being kept closed by the thumb. When the thumb is removed, the liquid in the closed arm should rise to a new level. This remaining gas should be brought around into the open arm, the opening closed by the thumb, and then released over a burning match or gas flame. An explosion indicates hydrogen. The gas in the closed arm should be about 20 to 70 per cent of the length of the closed arm; the absorption should remove about one-third (CO_2), leaving two-thirds (hydrogen). The gas, therefore, would have approximately the formula:

$$\frac{H_2}{CO_2} = \frac{2}{1}$$

If the above tests are positive, the organism may be assumed to be of the *Bacillus coli* group. Several organisms give these reactions and further tests are necessary to separate the most common, *B. coli communis* and *B. coli communior.*[19]

The gas-forming types are divided into two groups: the *B. coli* type with a low gas ratio, the predominant form in feces, and the *B. ærogenes* type with high gas ratio, the characteristic species on grains and grasses[38] and in milk.[20] The *colon-typhoid* group has been monographed recently by Weldin.[36]

Smith[34] is responsible for the proportions of CO_2 and H_2 usually given in the text books; they are formed from glucose and collected in the U-shaped tube which he suggested (Fig. 96). The proportion, 1 part of CO_2 to 2 parts of H_2, varies if the gases are measured at different times or with different strains. Bennett and Pammel[3] demonstrated that the first gas formed from glucose was principally H_2 and that the percentage of CO_2 was dependent on the amount which the culture medium had absorbed, so that the age of the culture had much to do with the proportions. The real percentages of the various gases formed can be determined only by drawing off the gas, by means of an air pump, from the closed arm of the tube.[21,32]

B. coli destroys sugars such as dextrose or lactose and forms three groups of products from glucose:[14,15]

1. Lactic acid.
2. Acetic acid, alcohol, succinic acid.
3. Carbon dioxide, hydrogen, formic acid.

The formation of the lactic acid is not correlated with the other groups but the two latter groups are closely related. The addition of calcium formate stimulates a greater production of carbon dioxide and hydrogen.

Harden and Walpole[17] demonstrated the numerous products which this organism will produce in glucose and mannitol.

Products of the fermentation by *B. coli*	Glucose	Mannitol
Alcohol	12.8	28.1
Acetic acid	18.8	9.5
Lactic acid	31.9	18.6
Succinic acid	5.2	8.9
Formic acid	0.0	3.0
Carbon dioxide	18.1	28.4
Cubic centimeters CO_2 formed per gram	91.8	143.0
H_2, cubic centimeters per gram	110.0	167.0
Ratio of H_2 to CO_2	1.19	1.18

The chemical products of the fermentation of glucose by *B. coli* would indicate a reaction somewhat like the following:

$$2C_6H_{12}O_6 + H_2O = 2C_3H_6O_3 + C_2H_4O_2 + C_2H_6O + 2CO_2 + 2H_2$$

Glucose + water = lactic acid + acetic acid + alcohol + carbon dioxide + hydrogen.

The reactions and the products of fermentation are the same with levulose.

Various carbohydrates, such as dextrose, levulose, mannitol, rhamnose, arabinose, xylose, have been tried by Winslow, Kligler, and Rothberg[39] as sources of carbon for a number of strains of *B. coli* and the colon-typhoid group, arranging them according to their fermenting ability (Fig. 97). The first and simplest are those strains able to ferment only hexose sugars; the second group, mannitol also; the fourth group adds arabinose and rhamnose; the fifth group includes xylose; the eighth group,

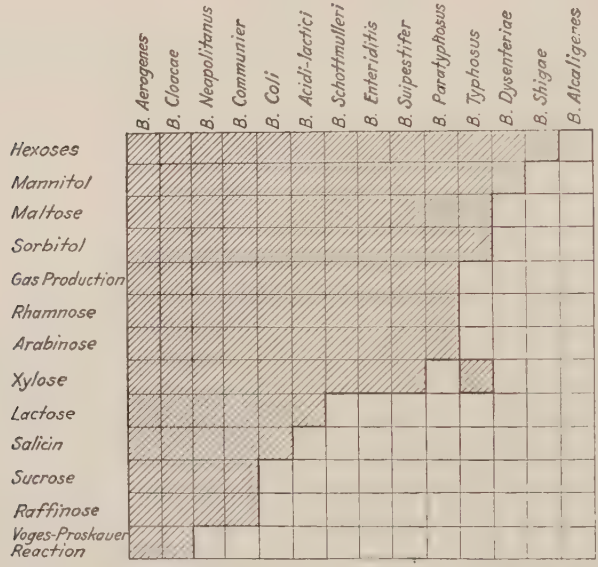


Fig. 97.—Reaction of members of *B. coli* group to carbon compounds; sugars fermented are shaded. (*From Winslow, Kligler, and Rothberg.*)

lactose; the last group lactose, and all the simpler carbohydrates, with the formation of two parts (approximately) of CO_2 to one part of H_2.

The amount of the various chemical compounds produced by the fermentation of glucose by *B. coli* varies widely. Variations in the amount of oxygen change the percentage of lactic, acetic, and succinic acids as compared to the hydrogen, carbon dioxide, and formic acid, but will not change the percentage of alcohol.[14] Besson, Ranque, and Senez[4] found gas and acid production to commence after the organisms have ceased to increase in number, the acid forming rapidly after being started, about 50 per cent of it in the first hour.

The organism destroys these sugars by the action of enzymes. Grey[14] suspects that a number of enzymes may be involved, some producing the group of products, succinic acid, acetic acid, and alcohol; while another enzyme may be responsible for the lactic acid. Some complex products may be formed in the early stages of growth to be later broken down into other simpler ones.

The organism. B. coli is a short rod 0.5 to 1.0 micron in breadth and about 2 microns in length. The organism is motile, when young, by means of flagella attached around the body (Fig. 98).

While *B. coli* reproduces by lengthening the individual cells and then splitting them into two of approximately equal size, Mellon[26] claims that this usual snapping into two fragments does not always occur, but, at times, typical budding takes place, such as is characteristic of the yeasts (Fig. 99). No explanation is offered for this occurrence but it is to be considered as "anormal." Similar observations on budding have been made on the related organism, *B. typhosus*.[18]

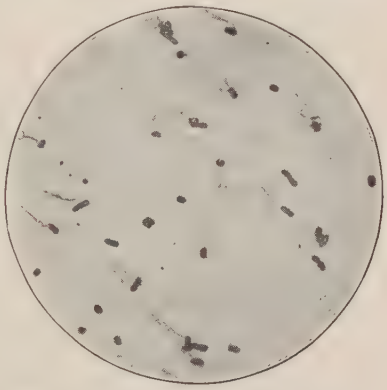


FIG. 98.—*B. coli* with stained flagella (*From Kral Institute.*)

The Physiology of the Organism.—In gelatin culture the growth is largely at the top of the gelatin, but a fine growth follows the entire line of puncture, indicating that oxygen is not necessary for its growth but that it thrives a little better where it is available. The gelatin is not liquefied.

B. coli grows very quickly in milk or in broth. In milk, an acid coagulum is formed. In broth, the growth is very abundant and is spread evenly throughout the tube. The rapidity with which *B. coli* grows in nutrient solutions without organic substances is much less than if some of the vitamin-carrying compounds are present. The addition of vitamins to a solution containing only mineral salts will more than double the growth.[9]

Protein Destruction by B. Coli.—These intestinal organisms are of the proteoclastic types and may, at times, split up the proteins into amino acids; even in apparently

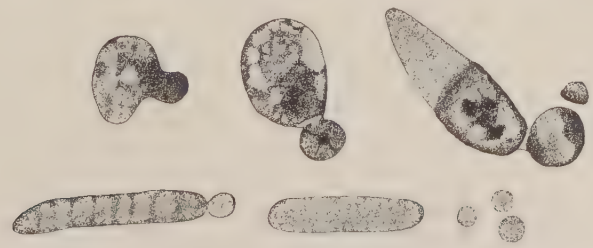


FIG. 99.—Abnormal reproduction in *B. coli*. The organisms at times bud smaller bodies by a process very similar to that found in yeasts. (*After Mellon.*)

normal stools the fecal organisms produce toxic amines.[16] Of the 26 stools studied, 18 were normal; 16 of the 26 stools, or 62 per cent, showed decarboxylated histidine; 17 of the 26 (65 per cent) showed decarboxylated tyrosine; and 12 of the 26 (46 per cent) both histamine and tyramine.

Chemical Conditions for Indol Production.—One of the very marked characteristics of this organism is that many strains of it will break up peptone into *indol*, one of the intermediate products of protein decomposition. The production of indol is important in the intestine because it may be absorbed and act as a poison, causing auto-intoxication. Met-

chnikoff[27] believed that too much absorption of putrefactive substances would lead to premature old age, but indol is not one of them. The production of indol is dependent on the absence of sugars from the medium, for the organism will use the sugars and form no indol.[31] Distaso[10] secured indol in sugar broth, providing he used tryptophan instead of peptone. Tryptophan, being a simpler compound than the peptone, is more readily attacked. The loss of the power to produce indol in old cultures is the result of their inability to take the first steps in the digestion of complex proteins. If one of the sugars that are very difficult to ferment (such as maltose, saccharose, or dulcite) is substituted in the culture medium for the more readily destroyed glucose, lactose, or mannite the *B. coli* will produce indol.[33] The acid formed from the sugars after fermentation checks growth and indol production,[40] the medium must be neutral. It would seem, therefore, that indol production is dependent on a vigorous culture; on the presence of peptone (or, preferably, the more easily digested tryptophan); and on the absence of easily fermentable sugars such as glucose, fructose, or lactose.

Variability in Indol Formation.—Cultures of *B. coli* when first isolated usually form indol, but the loss of this ability is a common change in old laboratory cultures. The fact has already been noted that the addition of tryptophan will induce indol formation, and it is also possible to induce its formation very easily[31] by adding the enzyme trypsin to the ordinary peptone broth in which most indol tests are made, the trypsin attacking the peptone and the resulting amino acid being easily fermentable. This result has been confirmed,[23] but indol production cannot be stimulated by the addition of tryptophan to strains which do not already produce it. Old cultures apparently lose some of their proteoclastic enzymes.

In many strains its production is very weak and indol is not a constant character even in strains where it is strongly marked.[12] Continued cultivation in a medium containing 0.05 per cent of phenol develops strains which do not produce indol, although the original culture may have been a strong indol former. Indol-producing strains seem to be inhibited by the phenol to such an extent that a culture finally arises which produces no indol.

VARIATIONS AND MUTATIONS IN BACILLUS COLI

B. Coli is Really a Group of Organisms.—Variability as to sugar fermentations, indol formation, nitrate reduction, and size has been the subject of intensive investigation in recent years. Sugar fermentations have been much investigated, since organisms of this group are very ready destroyers of the common sugars. Massini[25] discovered that if *B. coli mutabile* were plated out, daughter colonies appeared on the plates which would ferment lactose, although the parent strain and the majority of the colonies would not do so. Klein,[22] who investigated strains of this sort, seems to regard them not as *mutations* but as throwbacks to an ancestor that fermented such sugars. Such variations, usual in strains isolated from the urine or feces of horses, may have had their powers of fermentation altered by passage through the animal body.

After growing for some time on culture media in the laboratory, the fermentative power returns to a certain portion of them, although a large percentage of them never regain it. In Klein's experiments, over 50 per cent regain this character in cultures, so he cannot consider it as a mutation similar to that in the higher plants, where only about 1 to 3 per cent of mutants appear. Massini's discovery created much interest at the time, as the deVries theory of mutation was being actively discussed, and it was felt that, perhaps, even bacteria occasionally changed their characters in sudden and pronounced manner. It would seem probable, however, that the organism is very adaptable but that its adaptations are not usually permanent.

Variation of Strains of B. Coli to Heat.—Gage and Stoughton,[13] with a freshly inoculated and apparently pure culture of the organism, found that, while nearly all were killed by the temperatures at which bacteria die when exposed in tubes of liquid media, *i.e.* 55 to 60°C., plates made from cultures heated up to 80°C. showed that some individuals had survived. The experiments seemed to show the possibility of developing a race which would stand a very much higher degree of temperature than the ordinary *B. coli*.

The variation which occurs in nature is undoubtedly due to the substratum upon which the organism happens to be growing at the time it is isolated. Winslow, Kligler, and Rothberg,[39] in their study of the relation of the various members of this group to the various carbohydrates, did not question the constancy of the reactions nor make attempt to adapt the various species to forms of carbohydrates upon which they usually did not seem to grow well. Copeland and Hoover[7] isolated 3,000 organisms resembling *B. coli*, in that they fermented a lactose-bile solution, but only 65 per cent of them gave positive tests in milk, nitrate solution, peptone solution, and gelatin, 28 per cent failed to produce indol, and 5 per cent failed to reduce nitrates, all standard characteristics. We are at a loss, therefore, to describe the exact characteristics of *B. coli* from a physiological standpoint; its physiological characteristics change under cultural conditions, or the substratum upon which the organisms are growing favors some individuals.

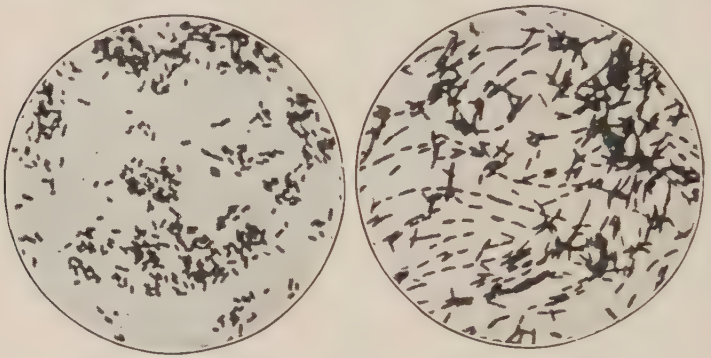
The *B. coli* strain of each individual differs from that of every other person, both in cultural and physiological properties, and, also, in the antagonism which it exhibits to other strains.[28] The interesting suggestion has been made that criminals could be identified by the particular strain of *B. coli* which they carried in their intestines. The possibility also exists that a change in the strain of *B. coli* of any individual could be effected by an alteration of the diet and inoculation with another type of organism.

Morphological Variations in B. Coli.—The morphological description of the organism is that it is a short rod, 0.5 micron in width and 0.6 – 2 microns in length, motile by flagella placed all around the cell (Fig. 98). Extreme variations in length are very noticeable in ordinary, newly isolated cultures. These variations suggested to Barber[1] that it might be possible to isolate (1) a long, filament-forming and (2) a short, single-celled race by the use of his apparatus with which a single organism or thread could be selected and removed from a hanging drop (Fig. 100). By selecting a filament and single cells from the drops, Barber developed one strain which produced filaments, a characteristic which seemed to be constant; one with short single cells; and, from individuals which were not motile, was selected a strain which lacked motility.

Motility is not a constant character in this organism and it would seem, from Barber's experiments, that some pure cultures might show it and others might lack it,

depending upon the individual which happened to give rise to the colony and to the resultant pure culture. Barber gives no measurements of the length of the cells, but his photographs would indicate that the filament-forming type is made up of longer individuals. In cultures, the two strains were distinct, the colonies of the filament formers being different in surface growth from the ordinary types.

The Lag Period and the Growth in Culture.—The period of slow growth in culture media, immediately after transfer, is known as the period of *lag* and is, to quite an extent, dependent on the age of the culture, since young cultures are more likely to



A *B*

FIG. 100.—Variation in *B. coli: A*, parent types from one day old broth cultures; *B*, new race isolated by the pipette method and grown under similar conditions. (*From Barber.*)

show a shorter lag period than older ones, probably due to the formation of acid. *B. coli*, being a rapid and prolific grower, has been studied in this respect,[30] with the following results:

TABLE SHOWING THE EFFECT OF AGE ON THE INCREASE IN A BACTERIAL CULTURE AFTER TRANSFER

Age of parent culture	Number of bacteria in one drop of the subculture after the following minutes						
	0	60	80	100	120	180	300
17 hours	29	38	45	88	116	728	41,307
4 days	28	28	26	27	29	136	7,984
12 days	18	18	19	19	12	22	3,623

Transfers from young parent cultures gave more organisms, and much more quickly, than transfers from old cultures. The transferred organisms themselves contain some hindering chemical which must be excreted from their bodies or be neutralized before full assimilation and growth can again set in and division attain a maximum; the transfer may carry over into the new media enough material from the old culture to act as a deterrent to growth until it becomes neutralized; or, the cells may lack a normal amount of CO_2.[29]

Old Cultures and the Checking of Growth.—The questions arise: What is the cause of the stoppage of bacterial growth in an old culture? Why does the growth cease after a time even with food still abundant in the medium? Conradi and Kurpjuweit[8]

found that *B. coli* developed in the medium a substance which checked the rapidity of growth if added to other fresh media. The antiseptic chemical excreted by the bacteria is comparable to carbolic acid in its effects on growth; for, while the latter has an antiseptic influence only in a dilution of 1:1,250, this substance checks growth when diluted 1:3,200. It is impossible to separate it out with strong alcohol or by centrifuging, but heating or boiling cultures destroys it, and it will not pass through a fine Berkefeld filter.

They are of the opinion, therefore, that it is so intimately connected with the life of the bacteria that it is almost impossible to separate it from their bodies, seeming to be produced as the organisms grow, the quantity depending on their life activities. Eijkman[11] claims that on agar or gelatin, bacteria such as *B. coli* form abundantly a toxic substance, the nature of which is not known but which will not readily pass through a Pasteur filter, although it will diffuse through a membrane and is easily destroyed at a temperature which will kill the organisms themselves. This deterring agent acts most noticeably on organisms from the cultures which produced it and, to a less extent, on similar bacteria. Intestinal bacteria such as *B. coli, V. choleræ*, etc., grow sparingly on agar cultures containing the filtrate from feces, but if this filtrate is first heated to 50 to 60°C., no effect on the growth is noticeable.

The nature of this growth-deterring compound or compounds was not determined by Eijkman, but similar results have been obtained in fungi from using old "staled" media. The retarding substance was sometimes partially volatile and a medium could be renovated by boiling or by the addition of alkalies or acids to neutralize it.

On the other hand, Manteufel[24] is convinced that no such thermolabile substance exists in old cultures and that the death of organisms in cultures of broth, agar, or gelatin, as well as in the intestines of the animals where they occur, results from rendering valueless the substratum upon which they have been growing.

The Increase from Single Organisms in a Fresh Culture Medium.—Barber[1] studied the growth in new cultures, using his apparatus, carrying a single organism over into a new medium where the rate of growth could be watched hour by hour. The individuals continued to divide when the temperature was kept as low as 10°C, and the maximum number of divisions occurred at 37°C. when a new generation appeared every 17 minutes. This high rate of division continued almost to 45°C. when it would fall away sharply, and at 49°C. no growth could be observed. Motility and rapidity of growth do not seem to be associated.

The Importance of B. Coli in Water.—This organism has been studied so intensively because it is used in many countries to detect the contamination of drinking water by sewage of human origin. Every gram of sewage contains the colon organisms by the million, so that it can be found wherever sewage has made its way into river or lake water. The typhoid or the cholera organism is very difficult to isolate from water, as it is present only in small numbers as compared to *B. coli*, so that it is much easier to determine the presence of the latter and pronounce the water contaminated and *possibly* dangerous than it is to isolate the disease organism itself. Water is condemned usually, therefore, on suspicion, and not because a specific disease organism has been isolated. The methods used and the relation of these organisms to water supplies will be discussed more in detail in the chapter on Bacteria in Water.

The continued existence of *B. coli* in water which has been inoculated with it, is dependent on the dissolved substances. The best liquid for its continued survival

seems to be distilled water. Busson[6] placed *B. coli* in distilled water in a large test tube which he closed by sealing the glass. This tube was kept at laboratory temperatures in the dark and samples were taken from it every 3 or 4 months for testing, the tube being closed again with sealing wax. This testing was repeated until the cultures were 6¾ years old at which time they continued to give abundant pure cultures on gelatin plates and the strains isolated showed all the cultural characteristics of the original. 98 to 99 per cent of the gas-forming organisms die off in water in the first 10 days, the *B. ærogenes* type being somewhat more resistant than the *B. coli* type.[38]

These results with *B. coli* are similar to those already obtained by Bolton.[5] who inoculated distilled water with various typical aquatic and disease-producing varieties of bacteria and kept them at 22°C. He found an actual increase for 36 hours after inoculation, with a gradual dropping off after that time for the water types, but pathogenic species died within a day or two. The water bacteria could still be found after 7 months, 540 to 760 colonies per cubic centimeter. The explanation given by Bolton for the persistence of the water forms is that, in ordinary methods of inoculation, enough organisms are introduced to furnish food for a number of generations of their descendants, and, when it is remembered that the actual weight of 20,000,000 of bacteria is only $\frac{1}{100}$ milligram, it will be seen that not very much food is necessary to carry them along. The persistence of bacteria in drinking water and sewage is a problem which will be discussed in connection with water bacteria.

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CHAPTER XXII

BACILLUS AMYLOBACTER (AN ANÆROBIC ORGANISM)

(*Granulobacter sacchrobutyricum, Amylobacter butylicus, Clostridium Pastorianum, Plectridium plectinovorum, Granulobacter pectinovorum*)

Pasteur[22] described the first organism, *Vibrion butyrique*, which was able to live in the absence of air and produce butyric acid by the destruction of sugars. Pasteur covered the culture media, in which he had these organisms growing, with a layer of oil, securing approximate anærobic conditions.

A similar organism, or varieties of it, has been investigated repeatedly and in almost every instance has been renamed. As Pasteur gave practically no morphological description, the first account of this bacillus must be considered to be that of Van Tieghem[26] who described it as *Bacillus amylobacter* by which it is generally known in bacteriology. The most complete investigation of the group of which it is a member is by Bredemann;[5] the various members of the group have not been described systematically, so that it is difficult to decide which member is under discussion.

Isolation of the Organism.—This organism is abundant in the soil, Gaffy[11] being the first investigator to isolate it from the inside of potatoes. It may be secured directly from the soil, the following method of enrichment[16] insuring a rapid growth and an almost pure culture at once. A medium-sized unwashed potato is pierced two or three times with a scalpel and covered with water in a beaker.

The beaker is covered (to keep the odor inside the vessel) and set in the 37°C. incubator for 2 days. At the end of this time, if a drop of material from the inside of the tuber is examined, an abundance of slender motile rods will be found, many of them swollen at one end from the development of spores. These swollen ends (drum-sticks) gave it the old name by which it was known, *Clostridium*.

The organism may now be isolated in pure cultures by the use of the dilution method, followed by the growth of the organism under conditions where the oxygen supply is kept at a minimum. A piece of glass tubing about 15 centimeters in length and of an outside diameter of 1 centimeter is plugged with cotton at both ends and sterilized in the dry-air sterilizer at such a temperature that the cotton is slightly browned. The tube is opened at one end by removing the plug of cotton and a little sterile agar poured into it, sufficient to fill 1 to 2 centimeters of the tube. A loop of material from the inside of the potato is put into a test tube of water and then a few loops of this dilution into a tube of melted agar. Enough of this agar containing the organism is poured into the culture tube almost to fill it and it is then replugged with the cotton. The tube is incubated at least 2 days at 37°C. and then examined for colonies in the agar. If a colony is located not too near to other colonies, the tube may be filed and the glass broken near to it. The Burri tubes may be used, the

procedure being practically the same. The colony may then be transferred to a tube of agar in a stab culture.

The Organism.—The organism is a long, slender rod, actively motile (Fig. 101) by means of 8 to 10 cilia.[2,5] It usually occurs singly but at times in pairs. Inside the organism, before spore formation, numerous small bodies (granulose) are scattered through the cell; these inclusions stain blue with iodine and have given the organism still another name, *Amylobacter*. Grown in the presence of small amounts of oxygen, the organism stains yellow with iodine.

The spores begin to form while the organism is still motile. While the spores do not readily stain in aqueous solutions of the aniline dyes, they take the weak iodine-potas-

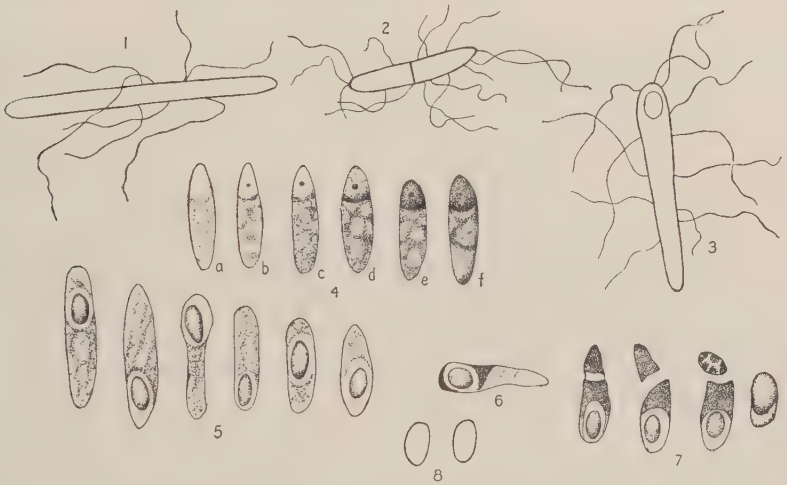


FIG. 101.—Stages in development and life history of *Bacillus amylobacter* A. M. et Bredemann: 1, vegetative form showing arrangement of flagella; 2, same, division; 3, flagellate form containing spore; 4, development of spore by accumulation of material around a dark-staining granule; 5, usual spore forms; 6, spore formation with accumulation of material outside spore in shrivelled remains of organism; 7, splitting of bacterium with development of a part as a spore capsule; 8, spore still enclosed in old bacterial wall.

sium iodine solution of Arthur Meyer[19] (p. 152). An intine and an extine can be readily seen after this stain. The average size of the spores is 1.18 × 2.11 microns. These spores germinate readily, first swelling and then a rod about 0.6 to 0.8 micron in diameter issuing from them. They grow in length so that at the end of 16 hours on agar 28°C. they are 1.0 to 1.2 × 3 to 12 microns in size. A chain of organisms is not usually germinated from a spore. The young bacteria soon acquire flagella, some of them carrying around with them, for a time, the old spore case out of which they have emerged.

The organism stores, as reserve food, the carbohydrate compounds, glycogen and iogen (granulose). As in the yeasts, the glycogen stains with iodine, giving a somewhat different color (red-brown) from that obtained with starch of vegetable origin, but the iogen (of Bredemann) gives a typical blue reaction. Pasteur, who probably first discovered this organism, noted its reaction to iodine, and Van Tieghem gave it the name *Amylobacter* because of its uniqueness. Every investigator

who has studied its morphology has interested himself in this deposition of carbohydrates in the cells.

Glycogen is scarce in the thin rods but, as they begin to thicken, they become filled with granules which extend to the poles. This accumulation begins to disappear with the spore formation; at the completion of this process, the cells are more or less free from it again.[5] In some spores this same material is stored, but they may be pathological, as their germination has not been observed. In fact, only about 2 to 3 per cent of the spores ever germinate even under conditions favorable for growth.[9]

At times, the cultures show short, coccus-like disintegration pieces among the cells themselves, called, by Arthur Meyer, "microoidia," and observed by Garbowski[12,13] in other bacteria. The microoidia undoubtedly arise from the division of the ordinary forms grown in culture media not especially adapted to them, such as that which lacks calcium carbonate to neutralize the acids formed as growth proceeds. The fragmentation is usually by cross-walls, but Bredemann believes that, at times, they divide in two planes in a manner similar to the micrococci. These *microoidia* were formerly believed to be incapable of further development, but Bredemann transferred them to fresh tubes of agar and found that they developed into ærobic colonies. In other words, this anærobic species develops under special conditions into a coccus-like form which continues as an ærobe. In all strains studied by Bredemann, these microoidia appeared in the same manner.

Such an unusual occurrence would need confirmation, but numerous similar cases have been reported in bacteria.[17] Cunningham and Jenkins[8] have observed coccus forms in 30-day old agar cultures of *B. amylobacter*. They claim to have excluded all possibility of contaminations. The cocci came from the breaking up of cultures of the motile organism, were ærobic (as Bredemann claimed), and did not fix nitrogen in a soil extract containing dextrose.

THE BUTYL ALCOHOL AND ACETONE SPECIES

Beijerinck[3] isolated from ground grain an organism which he named *Granulobacter butylicum* which produced butyl alcohol in such quantities that it could be used as a commercial source for that chemical. This organism was probably only one of the *Bacillus amylobacter* group with the special property of producing butyl alcohol in quantity. He noted intermediate forms among the species which he attempted to make from his individual isolations. This organism produces butyl alcohol, hydrogen, and carbon dioxide from maltose through the action of enzymes which it secretes; diastase for the solution of the starch of the barley meal into maltose; maltase which converts maltose into butyl alcohol; dextrinase which converts dextrin into maltose; and various other types of diastase, but no enzyme which acts on glucose. Since *G. butylicum* is very abundant on the outer coats of grains, it is only necessary to add enough boiling water to some unsifted barley meal to make a thick gruel and allow it to incubate for a day or two at 37°C. when butyl alcohol can be detected by its odor. This organism will be found so to predominate in the gruel in a few days that it can be used for commercial production of butyl alcohol.

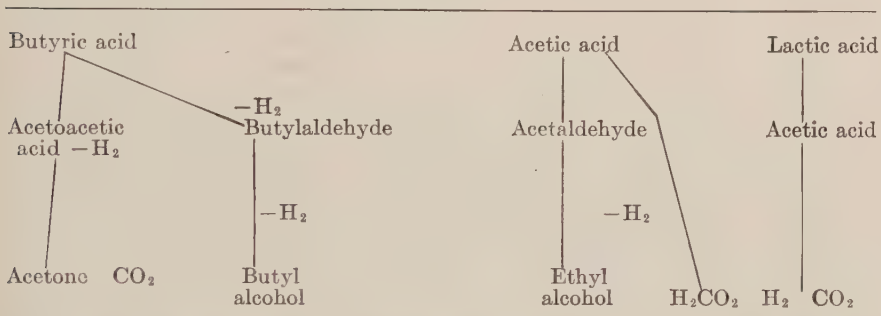
The Materials Produced in the Fermentation.—The butyric acid produced by the fermentation process has the formula: $C_4H_8O_2$, the structural formula being $CH_3 - CH_2 - CH_2 - COOH$; the acid known as "propyl carboxylic acid."

The formula for the fermentation of one of the hexose sugars would be as follows:

$$C_6H_{12}O_6 = 2H_2 + 2CO_2 + C_4H_8O_2$$

But it is hardly probable that the equation is nearly so simple. Beijerinck was certain, however, that carbon dioxide was always produced by the organism, and, usually, hydrogen; also that the butyl alcohol was converted into butyric acid by the process of oxidation.

More recent investigations of the products of this organism would seem to indicate that a series of intermediate products occur with butyl alcohol and acetone as the more important end products. Speakman[25] found that the non-volatile acid produced from the starch and sugar is lactic but that acetic and butyric are first evolved in the fermentation of bran mash. He suggested the following series of compounds:



The end products of such an equation would be acetone, butyl alcohol, and ethyl alcohol.

After continued growth for some time in a mash with the formation of butyl alcohol and acetone, the activity of these organisms becomes somewhat weakened. Their full activity can be restored by pasteurizing the mash containing them after they have all sporulated. The vegetative organisms which come from the spores which have escaped pasteurization are much rejuvenated and the solvent production of a strain has been increased 100 per cent by repeated pasteurization after sporulation.

The butyl alcohol organisms are not at all susceptible to butyl alcohol itself or to butyl compounds, such as butyl resorcinol, to which many common spore-forming types, such as *B. subtilis*, readily succumb. Butyl alcohol-formers seem to have developed an immunity to the butyl radical.[27]

The Amylobacter group may be divided into two sections (1) butyric and acetic producers; (2) butyl alcohol and acetone (both neutral in reaction) producers.[18] With the latter group, 1.8 grams of glucose yielded 0.155 gram acetone and 2 grams of starch gave 0.11 gram acetone.

This Organism Grows in a Minimum of Oxygen and Fixes Atmospheric Nitrogen.—*B. amylobacter* is of importance and has been much studied for two reasons: (1) it can live without oxygen; (2) it will fix atmospheric nitrogen and combine it in its protoplasm.

Growth without Oxygen.—Pasteur was interested in the organism which he found because of the fact that it could live without air; a revol-

utionary thought, both from the standpoint of respiration and fermentation, which, like many new theories, met with determined opposition. His methods were so carefully studied and executed that, after the lapse of over 60 years, we can admire their simplicity and accuracy.

From a very simple observation, he had arrived first at the conclusion that these organisms did not need oxygen. If some of these motile forms are placed on a cover glass, those at the center of the drop will be found in active motion; those on the margin where the air comes in contact with them are immotile, with various gradations between the two extremes where motion is slowing up. He grew them in cultures where air was absolutely excluded by a three-parted tube and no oxygen was introduced in the inoculation process, but still the colonies increased in size and the organisms produced the characteristic butyric-acid fermentation. So Pasteur added a new saying to microbiology: "Fermentation is life without air."

In spite of active opposition by many of the ablest biologists of his day, he was able to win the scientific world over to his viewpoint. One of the last of his critics was Gunning of Amsterdam, of whom Clark[7] says:

"Let us turn back to a scene in the French Academy of July 1st, 1878. On that day Gunning of Amsterdam presented in full his arguments against the existence of anærobiosis. At the close of his remarks Pasteur rose to say that for 16 years he had been seriously concerned with the source of error noted by Gunning, and had concluded that his cultures were free from molecular oxygen, because indigo-white remained reduced. This statement we can now interpret. In aqueous solutions electrode potential differences such as those of the indigo system can be interpreted in terms of the hydrogen electrode and the pH we can calculate for a partially reduced indigo solution the hypothetical hydrogen or oxygen pressures in equilibrium with the system. In a case where we allow bacterial reduction of indigomonosulphonate to proceed to 80 per cent reduction we measured the potential electrometrically and calculated therefrom the oxygen pressure. It came to 10 to 36 atmosphere. Taking the data of Millikan on the number of molecules per gram of molecular weight of gas we calculate that less than one discrete individual molecule of oxygen was present at equilibrium in 10 to 13 liters of the culture. To speak colloquially, that is some anærobiosis!"

Pasteur was very certain that these butyric-acid organisms would grow in a medium absolutely free from oxygen, and divided bacteria into two groups, those growing in an atmosphere containing oxygen to which he gave the name of *œrobes* and those growing in the entire absence of oxygen, *anœrobes*.

We have learned since, however, that such sharp distinctions are not tenable, since many organisms are intermediate in their oxygen requirements, many can adapt themselves to one or the other condition, while even the obligate forms of one or the other types can stand the lack of oxygen, or adapt themselves to live for a time in oxygen. Beijerinck's experiments[2] were very simple but illustrate the points in question:

The organisms to be tested are placed in a drop of water and covered with an ordinary, round cover glass (Fig. 102). One side of the cover glass is now raised with a forceps and a little U-shaped piece of platinum is placed under one edge so as to tilt it slightly. The air comes under the glass as a diameter to the circle of the glass from whose margin the oxygen gradually becomes scarcer and scarcer. If an organism requiring oxygen such as *B. fluorescens liquefaciens* is placed in the drop, the figure produced will be that of 3. The active organisms will swim to the margins of the cover where oxygen can be secured, but at the center of the drop the bacteria that are incapable of motion will be forced to remain. Between these two groups will be a clear zone avoided by both groups. If an organism of the Spirillum type is

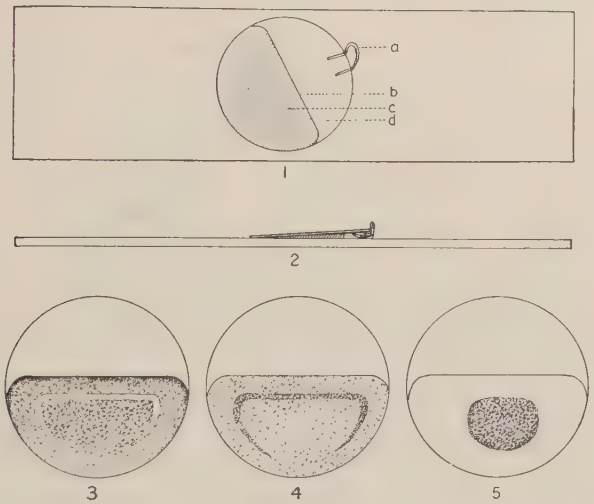


FIG. 102.—Arrangement of motile bacteria under cover glasses, according to oxygen requirements: 1, glass slide with circular cover glass supported at one side by small U-shaped piece of platinum wire, *a*, space at *d*; 2, cross-section showing arrangement as seen from the side; 3, respiration figure of ærobic type with bacteria collected next to meniscus of air; a zone free from bacteria and a central region filled with non-motile bacteria that cannot get into the better aerated region; 4, respiration figure of spirillum type with organisms at the center except a few that are not motile; 5, respiration figure of anærobic type of bacteria crowding away from aerated region into zone at center. (*After Beijerinck.*)

placed in the drop, it will place itself as far as possible in the region of least oxygen, forming a half circle at the center.

Beijerinck demonstrated this relation to oxygen (Fig. 103) by placing a little gelatin in a test tube, covering it with water and then inoculating the water with the organisms to be tested. Or, in some trials he used a very diluted agar (0.1 per cent) instead of the distilled water, this agar being so thin that the bacteria could find their way through it as though it were water, but the layers that form would not so readily be shaken up and lost. *B. prodigiosus*, a marked ærobe, finds its way, regardless of the fact that it is not highly motile, to the surface of the water. On the other hand, *B. coli* forms a double layer of organisms in the test tube; one just at the surface, and a second layer, which is much thicker, just a little below the upper one, but separated from it. *B. coli* grows on the top of an agar stab and also produces a fine line of growth which extends down into the agar to the bottom of the puncture, a so-called "wire-nail growth."

Beijerinck followed up these experiments with similar experiments[4] on supposed obligate anærobes, such as *B. amylobacter*. He was surprised to find that these organisms, instead of seeking the lowest portion of the cultures where the oxygen was the scarcest, formed a layer at some distance from the top, as though they found a certain amount of oxygen advantageous. These organisms, therefore, could not be considered as *ærophobes* but as *æromicrophiles*, that is to say, they were not *haters of oxygen* but *likers of a little oxygen*. In spite of this fact, they can, if necessary, grow and increase in numbers in media without the slightest trace of oxygen.

Pasteur had pointed out that the effect of the presence of oxygen was to stop growth and to check the motility of the organisms. The question of the death of the organisms from their exposure to oxygen was left an open one, which Chudiakow[6] attempted to answer experimentally:

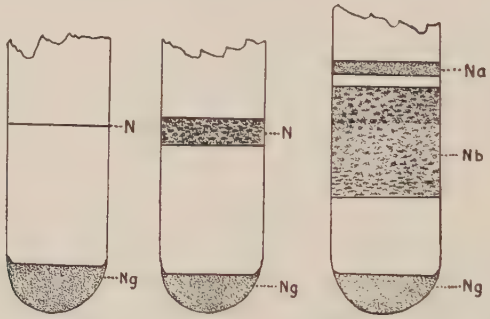


FIG. 103.—Planes of bacteria due to their reaction to oxygen. *Ng* layer of gelatin; distilled water above; 1, *Bact. zopfii*, an anærobe; 2, *B. prodigiosus*, an ærobe; 3, *B. coli communis*, a facultative anærobe, with a double plane, *Na* and *Nb*. (*After Beijerinck.*)

Tubes of agar heavily seeded down with non-spore bearing forms of the organism to be tested were left exposed to the air for a definite time and then removed to a vacuum; control plates were kept in the vacuum without being exposed at all to the air. This method worked well on *B. amylobacter*, the injurious effect of air and its killing powers being effectively demonstrated. One hour in the air gave very little difference in the exposed or control plates; about the same number of the colonies developed; 4 hours' exposure not only killed quite a number of the organisms, as shown by the colonies, but the colonies which appeared were smaller in size, indicating that they had been injured; 15 hours killed all the organisms and not a single colony appeared.

The spores of the anærobes are known to be very resistant to the action of the air and may remain exposed to it for months without serious injury, but 265 days in the air made a very noticeable weakening in the colonies formed from them.

In the vegetative condition, an exposure to the air of only 10 minutes is sometimes sufficient to kill all the organisms, but usually 1 to 2 hours are required.[1] The very young stages and the younger spore stages are not so sensitive; the mature organisms are the most susceptible. The spores live on agar in the air an average of 8 days under laboratory conditions, but at low temperatures such cultures remain viable after 500 days.

Dorner[9] places the time for injury in the air at 40 minutes for the vegetative and 3 hours for the spore stage.

None of the investigators explain the marked changes which the oxygen induces in the protoplasm of the bacilli. The suggestion that

increased oxygen leads to such an increased and abnormal respiration that the organisms, adapted to a slower rate, are destroyed by the sudden loss of material would seem to be plausible.

The Technic of Growing Anærobic Cultures.—The difficulties in handling the cultures of the anærobic types has induced investigators to spend much effort in improving them. Some investigators have exhausted the air by the help of vacuum pumps; others have replaced the air by inert gases, such as hydrogen; while others have absorbed the oxygen by chemicals, such as an alkaline solution of pyrogallic acid.[20] Kursteiner[15] used the absorption method (pyrogallic acid in a strong alkaline solution) in preference to all others for obtaining a definite vacuum.

How Much Oxygen is Necessary?—The later theoretical work on these anærobic species has been largely in the hands of Arthur Meyer[19] and his students, Wundt[28] and Bredemann,[5] who have developed the fact that the so-called "obligate anærobes" will thrive in a small amount of oxygen but that if the minimum dose is increased, the growth will fall off until it is nil. In special apparatus, devised by Meyer,[19] the organisms could be tested for their growth (1) in subatmospheric pressures, (2) at atmospheric pressure, (3) at superatmospheric pressures. Bredemann, for example, obtained for *B. amylobacter* the maximum, minimum, and optimum growth. These facts give, for this organism, an oxygen-toleration curve. He also obtained, for the same organism, the maximum of oxygen toleration for spore formation and for growth of vegetative cells, together with the minimum curve for spore germination, spore formation, and growth of the vegetative cells. Wundt[28] grew various organisms, some of which were ærobes and some facultative anærobes, at various pressures below and above that of the air and compared them with the growth obtained in air. None of the organisms which he studied were strict anærobes, or he would have been unable to get any growth with them with any oxygen. Some idea of the range of oxygen in which these organisms can work is shown in the following tables which summarize a part of the spore germination results of ærobes and anærobes, the germination in air being reckoned as 1:

	In partial vacuum			In lessened air			Air	With gas mixtures		Oxygen	With compressed air	
Oxygen in milligrams per liter	3	6.8	9.3	20	70	200	276	537	1,061	1,336	2,163	4,317
Bacillus lactis	0	0	0	1	1	1.3	1	1	3	2.7	0	0
Bacillus Ellenbachiensis	0	0	1.2	1	1.1	1.5	1	1	2	2	6	0
Bacillus subtilis	0	4	1.2	1	42.8	1.3	1	0.5	2	2	3	5
Bacillus mycoides	0	3	2.4	1	0.7	0	1	1	4	3	0	0
Bacillus parvus	3.5	4.2	1.6	0.6	1.5	1.2	1	1	2	3	3	5
Bacillus asterosporus	2	...	1.6	1.4	0.7	0.8	1	1.7	1.5	2.7	3	2.5

From this table it will be seen that many organisms have a very wide range of oxygen requirements for spore germination; that many of them can live where oxygen is at very low pressure and again in pressures that are almost twenty times that of the atmosphere.

The growth requirements are somewhat different from those needed for *spore germination*, although we should expect the two to be about the same. The following table gives the growth requirements of the same organisms as compared to 1, obtained in the air:

	In partial vacuum			In lessened air			Air	With gas mixtures		Pure oxygen	With compressed air	
Oxygen in milligrams per liter	3	6.8	9.4	20	70	200	276	537	1,061	1,336	2,163	4,317
Bacillus lactis	0	0	0	1	1.3	1.2	1	3.5	2.	2.5	0	0
Bacillus Ellenbachiensis	0	0	1.2	1	1.2	1.3	1	1	1.5	3.5	0	0
Bacillus subtilis	0	3	1.2	1.3	2.1	1.3	1	.7	1.5	1.8	3.5	0
Bacillus mycoides	0	3	2.5	1.	0.8	8	1	1	2.5	3.	0	0
Bacillus parvus	3	1.6	1.5	0.8	1.4	1.1	1	2.5	1.5	3.	2.	0
Bacillus asterosporus	2	1.8	1.5	1.3	0.8	0.7	1	1.3	1.3	2.5	3.5	0

The same general tendencies are to be seen in this table as in the preceding one, but the great range under which many of the organisms will continue to grow is the outstanding feature.

The spore formation under different oxygen pressures is indicated in the following table:

	In partial vacuum			In lessened air			Air	With gas mixture		Oxygen	With compressed air	
Oxygen in milligrams per liter	3	6.8	9.4	20	70	200	276	537	1,061	1,336	2,163	4,317
Bacillus lactis	0	0	0	1.2	1.4	1.4	1	0	0	0	0	0
Bacillus Ellenbachiensis	0	0	0	1.2	1.4	1.1	1	0	2	2	0	0
Bacillus subtilis	0	1.6	1.3	1.1	1.5	1	1	1	1.1	1.8	0	0
Bacillus mycoides	0	1.6	0	1.2	1.2	0	1	1.2	2	2	0	0
Bacillus parvus	0	0	0	1.2	0	1.2	1	1.6	1.1	2	0	0
Bacillus asterosporus	0	0	1.7	1.5	1.2	1.4	1	0	0	0	0	0

The results may be further summarized in the following table which gives a maximum, minimum, and optimum for spore germination, growth, and spore formation:

Organism	Spore germination			Growth			Spore formation		
	Minimum	Optimum	Maximum	Minimum	Optimum	Maximum	Minimum	Optimum	Maximum
Bacillus lactis	20	400	1,336	20	276	1,336	20	276	276
Bacillus Ellenbachiensis	9.4	276	2,163	9.4	276	1,336	20	276	1,336
Bacillus subtilis	4.3	400	4,317	4.3	276	2,163	4.3	276	1,336
Bacillus mycoides	4.3	70	1,336	4.3	60	1,336	6.8	276	1,336
Bacillus parvus	3	276	5,687	3	276	5,687	11.3	276	1,336
Bacillus asterosporus	3	100	5,024	3	130	5,024	4.3	276	1,336

These results indicate a great range of adaptability among organisms, many of which are facultative anærobes; any organism has a trend toward a life in little or in much oxygen, the amount required by the obligate anærobes being so small as to be almost unmeasurable.

B. amylobacter develops well if the pressure is lowered to 5 millimeters but growth is very weak and late in appearing. This small percentage of oxygen is used by the organisms and not taken up by the culture media nor passed off in the fermentation products. The so-called "obligate anærobes," therefore, lead a twofold existence; they can make use of small amounts of oxygen but they can also live by the fermentation of the substances in the media alone with absolutely no oxygen. Some anærobes can use 20 millimeters and others as much as 40 millimeters. *B. amylobacter* can be accustomed to larger doses of oxygen so that it will develop well in 50 millimeters but reverts to a smaller percentage if grown again in a vacuum. A race may be developed also which will thrive in an absolute vacuum. Bachmann[1] agrees with the original Pasteur definition of ærobes and anærobes in bacterial life as expressed in his later contribution.[2,3] Pasteur defines ærobes as organisms which need oxygen in their life processes while the anærobes are those which can dispense with oxygen. This definition of Pasteur's is scientifically accurate for it separates the bacteria into two classes: the one class known as the "anærobes," has *permanent* intramolecular respiration with permanent oxygen respiration, while the other group has the capacity for *temporary* intramolecular respiration with permanent oxygen respiration, the "ærobes."

Meyer, however, would draw a little finer distinction by making the following groups:[21]

I. *Anærobes*, those organisms which can dispense with free oxygen.
   a. Obligate anærobes, those which cannot live in the air, but which can live without oxygen.
   b. Facultative anærobes, those which can live either in the air or entirely without oxygen.
II. *Aerobes*, those organisms which find oxygen indispensable for their existence.

This classification seems to cover the organisms that have been investigated to the present time and has found general acceptance.

How Did the Anærobes Arise?—Frankland[10] suggested many years ago that the anærobic types were developed from ærobes by natural selection due to an occasional lack of oxygen; organisms able to exist under the new conditions started a new race able to maintain oxidation processes in spite of this condition. By repeated selection, some of them came to be able to function entirely without oxygen.

A number of investigators have tried to change anærobes into ærobes but with rather doubtful success,[14] for, while as in *B. amylobacter*, the oxygen tolerance can be increased from 5 millimeters pressure to 50 millimeters, the condition is not permanent and the cultures revert. A number of other trials have been made with various organisms, but the results seem still to be questioned. The development of a different phase in *B. amylobacter*, in which it becomes a coccus and an ærobe, is interesting but will have to be conclusively confirmed by a number of observers.

Food Used by Anærobes.—Anærobic organisms have a different effect on media from ærobic ones since the latter use the sugars for oxidation processes, but the anærobes, it would be expected, can make use of the sugars and proteins to build them into their body structures directly and respire intramolecularly.

B. amylobacter derives its energy from the change of lactose sugar into butyric acid, a synthetic process. Other anærobes can grow, however, in the absence of sugars by the destruction of proteins, so that sugars are not necessary for anærobiosis.[14]

The anærobic organisms whose protein metabolism has been studied were isolated[24] from cases of animal or human disease, particularly of the type known during the war as "gas-gangrene." As the name indicates, the organisms form an abundance of gas and grow anærobically in the deeper wounds. In such wounds, they must dissolve the proteins and possibly use the sugars of the blood in order to survive. In pure cultures, many of them readily split sugars to form gas and acid, while others are very weak in this respect; some destroy proteins readily, others less easily. In some forms, the ability to produce proteoclastic enzymes is combined with a weak sugar-splitting enzyme; in others, a weak proteoclastic ability is united with strong sugar-hydrolyzing enzyme production.

The Fixation of Atmospheric Nitrogen by B. Amylobacter.—The second interesting phase of this organism is that it can fix atmospheric nitrogen, utilizing it to build its proteins. Its abundance in the soil makes it an important factor in nitrogen fixation, practically no type or depth of soil being free from it.[5]

Old cultures gradually lose their ability to fix nitrogen, but freshly isolated strains combine it in abundance. Dextrose or other sugars furnish the energy for this fixation, 1 gram of dextrose yielding between 1.4 and 7.6 milligrams of fixed nitrogen. The addition of small amounts of a nitrogen salt, such as ammonium sulphate, will not check this assimi-

lation of atmospheric nitrogen, but larger quantities lead the organism to derive its nitrogen from the culture medium rather than from the air.

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CHAPTER XXIII

TWO COMMON SPHERICAL ORGANISMS

STREPTOCOCCUS LACTIS (THE SOUR MILK ORGANISM)

(*Bacillus lactis; Bacillus lactis acidi; Streptococcus lactis acidi*)

Streptococcus lactis (Lister) Löhnis is the most common of the organisms which sour milk. The fermentation which it induces in milk was one of the first to be studied in bacteriology and the organism was the first to be obtained in pure cultures. Lister[13] obtained pure cultures by diluting sour milk so that each drop contained a single coccus, then adding a drop of this diluted milk to a tube of sterile milk. The pure species which had grown in many of the tubes received the name *Bacterium lactis*. This organism, since it is slightly oval, is generally designated as *Streptococcus lactis*.

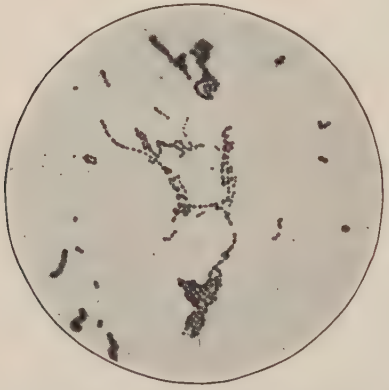

FIG. 104.—*Streptococcus lactis* from milk. (*From Kral Institute.*)

The lactic-acid organism is very abundant in the air of dairies (as Lister noted) and in the vessels in which milk has been kept. It rarely fails to be the predominating type, so that the souring of milk is to be regarded as a perfectly normal fermentation always to be expected.[10]

The organisms are very easily isolated from any sample of old milk if a little litmus solution is added to the agar before it is used for the plating. The small colonies with a zone of red around them are almost white in color, shaped like a double-convex lens if imbedded in the agar, but circular, and seldom exceeding 1 millimeter in diameter, if on the surface.

The Organism.—Some of the cells, short rods or nearly spherical, are 0.5 to 0.8 micron in breadth and 0.8 to 1.2 microns in length. While they may appear as single non-motile individuals, they are more commonly grouped in pairs, or occasionally, long chains are found (Fig. 104).

Lactic-acid Formation in Milk.—By the transfer of a portion of one of the lactic-acid producing colonies to a tube of neutral, sterile litmus milk, the milk will coagulate in about 24 hours when incubated at either 37°C. or at room temperature. The acid, which accompanies the bacterial growth, destroys the equilibrium in the colloidal condition of the milk and a curd is formed. In addition to coagulation, the litmus is reduced and a red surface ring is visible which broadens as oxygen is absorbed into the coagulum. The curd which is formed is practically without the formation of whey, a characteristic of the true lactic-acid organism. Lactic acid is the only acid

formed by this particular organism from the lactose in the milk, the chemical change being represented by the following equation:

$$C_{12}H_{22}O_{11} + H_2O = 4C_3H_6O_3$$
(Lactose) (Lactic acid)

The equation represents only the formation of the end product and a series of intermediate compounds may be formed, among which is probably pyruvic aldehyde.[21]

Part of this lactose is used in the respiration of the organisms and part to furnish energy for the upbuilding of the proteins, so that the amount of acid which may be obtained from a given amount of lactose is never exactly that which the equation indicates. Van Slyke and Bosworth[22] inoculated milk with a mixed culture of *Str. lactis* and *B. lactis aerogenes* and at the end of 60 hours found that 22 per cent of the milk sugar had been changed to lactic acid and that 88.5 per cent of the total amount of sugar had been converted into this acid. With cultures of *Str. lactis* in fresh pasteurized skim milk kept at 32.2, the relative amount of sugar converted into lactic acid is largest in the 10- to 24-hour period, although this time varies with the temperature used for incubation (Fig. 105). The amount of acid produced by *Str. lactis* rarely exceeds 1 per cent, the acid becoming noticeable to the taste when the percentage approaches 0.25 to 0.28. When the titrable acidity of the milk is 0.74 per cent, about one-half of it is found to be absorbed by the casein and remainder is free in the milk serum.

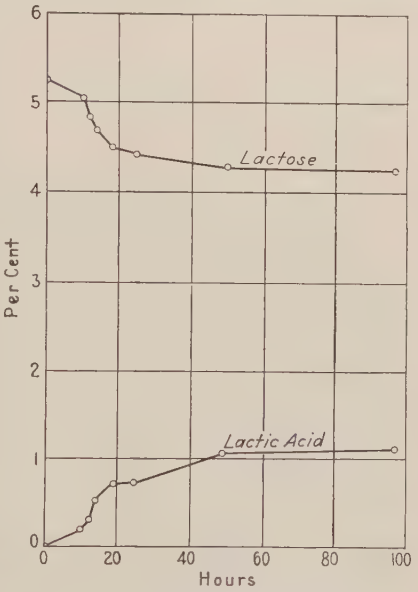

FIG. 105.—Formation of lactic acid in milk. The acid increases to about 1 per cent and stops but the milk still contains about 4 per cent lactose. (*From data by Van Slyke and Bosworth.*)

Theoretically, 342 parts by weight of lactose will yield 360 parts of lactic acid, but since the growth of the organisms is inhibited as the acid increases, over 4 per cent of the lactose usually remains in the milk. The growth of the organisms can be renewed several times by completely neutralizing the acid which has been produced. The lactose remaining in sour milk is utilized as the commercial source of practically all the lactose sugar on the market.

The souring does not destroy the food value of the milk since the caseins, albumins, fats, and all the lactose, except that which has been converted into lactic acid, still remain. Sour milk and fresh milk have just about the same food value. In addition to the souring, however, the lactic-acid organism suppresses the growth of other organisms which have a destructive action on the caseins and albumins in the milk. These organisms, many of which are derived from the air, soil, and manure, putrefy the milk and render it unsuitable for human food. The growth of these undesirable species is retarded by the presence of the acid. Many of the peoples of southeastern Europe, such as the peasants of Bulgaria, Rumania, Albania, etc., use the milk only after it has been soured, since souring insures that it is healthful.

Other Lactic-acid Bacteria.—The lactic-acid species found in milk are not the only organisms, nor are they even the relatives, of some of the other species which

form lactic acid, if the morphology of the organism is considered to be any measure of relationship. The *Lactobacillus delbrück*, used by the makers of commercial yeast cakes, is an example of a very high lactic-acid producer of the granulated, long, rod type found in cereals and on grains.[2] This organism will readily convert glucose or fructose into lactic acid while the disaccharide sugars are less readily attacked.

Another high acid producer, *B. bulgaricus* (Lactobacillus bulgaricus) originally isolated from the soured milk used extensively by the peoples of Bulgaria and Turkey, produces about 2.5 per cent of titrable lactic acid. It is the "organism of long life."[15] Similar bacteria are *B. bifidus* and *B. acidophilus*, the latter being used extensively as a therapeutic agent to change the character of the intestinal flora. It is given with 150 grams of lactose daily, this sugar (or dextrin) being essential to its establishing itself in the intestine as the dominant organism; or, it may be established by drinking 300 cc. of a milk culture daily. The intestinal bacterial flora then becomes *aciduric* instead of mixed.[10,18] The commercial cultures of these organisms are not always as represented; out of 107 samples examined by James[9] only 28 were reasonably pure and viable.

The entire group of lactic-acid forming bacteria has been studied intensively by Orla-Jensen.[16] His classification includes not only spherical forms but also true rod-shaped bacteria; but separates all the true producers of lactic acid from those which produce other acids in addition to lactic, principally acetic and carbonic. The group which produces the latter acids includes *B. coli* and *B. aerogenes*, both of which form large amounts of gas. These latter organisms are very abundant in the feces of the cow and find their way into milk if precautions are not taken.

The lactic acid produced, according to Orla-Jensen,[16] may be of the dextrorotary or the levorotary types, depending on the bacteria. If equal amounts of each are produced, the acid is of the inactive variety. One organism seems to produce one type of acid under all conditions and the type of acid formed cannot be altered by varying the conditions of production. He assumes that the production of dextrorotary or levorotary acids is the result of the work of special enzymes, one enzyme affecting only one type of sugar and being produced by the organism for that purpose. With *Str. lactis*, the acid is of the dextro type.

Uses of the Lactic-acid Organism.—The lactic-acid organism is used to sour milk for the production of butter and milk for cheese. The types of organisms which are found naturally in milk or cream do not give a product with a constant flavor, so that the producer of butter or cheese finds that the best method is to destroy them and replace them with a pure culture of lactic-acid formers: *a starter*. Butter or cheese produced from milk or cream ripened by these cultures is constant in flavor, but may lack the very fine quality which occasionally appeared in naturally fermented products, due to various aromatic acids.

Hammer[3] has found in the commercial starters, besides *Str. lactis*, other organisms which produce volatile acids which give flavor to butter made from cream soured with them.

Proteoclastic Enzymes.—*Str. lactis* will not liquefy gelatin if grown on gelatin plates as it does not secrete any quantity of proteoclastic enzymes. Some lactic-acid formers digest both peptones and casein[17] and the ripening of cheeses, such as Parmesan, is due to their activities. In cultures, *S. lactis* will grow for a time on agar but never profusely even with the addition of lactose, so that it would seem to be an organism which has adapted itself to milk as substratum and will not thrive well elsewhere.

The Souring of Milk during Thunder Storms.—The souring of milk or cream during thunder storms is not due to the action of the reverberations on the molecules of the milk but to the warm weather which usually precedes such storms. The high temperatures hasten the multiplication of the lactic-acid variety.

STAPHYLOCOCCUS AURANTIACUS (MICROCOCCUS AURANTIACUS)

The Orange Staphylococcus.—On Petri dishes, containing nutrient agar, exposed to the dust of the air for a few minutes, a number of orange- or lemon-yellow colonies will usually appear after being incubated at room temperature for a few days. These small colonies (1 to 2 millimeters in diameter) may vary in color from a cream or light yellow to a dark orange.

The Organism.—The spherical individuals are about 1μ in size; the grouping is in irregular masses varying from a single individual to twenty or more (Fig. 106). The groups are irregular in form, the organism dividing in all three planes to form them. Such a grouping of spherical organisms is the type of the genus, *Staphylococcus*, but some authors prefer the term *Micrococcus* for this non-parasitic group, reserving the former term for the organisms found on the skin and in pus.[1] There seems to be no real reason for such an arbitrary division and the group should be left intact until we know more about the life histories and relationships.

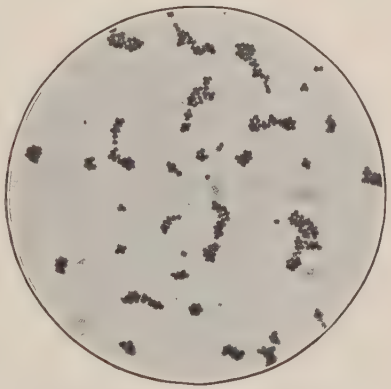

FIG. 106.—*Staphylococcus aurantiacus.* (*From Kral Institute.*)

Two common species are *Sta. aurantiacus*, an orange yellow and *Sta. aureus*, a lighter (golden) yellow. The variation in color is considerable, however, and other tests must, in many isolations, be made to determine the species.

These organisms are common in the air, water, milk, and soil. Milk seems to be the commonest habitat since most organisms find this substance a medium in which they may both survive and grow. They may be isolated not only from the milk itself, but also from the utensils, such as milking machines, connected with a dairy industry.[5,19]

The Colors and Coloring Matters.—The colors produced in the group of mass-forming cocci are all yellow, orange, or red; no blues, greens, or browns are known.

The amount of color produced is variable but seems to be most developed in media containing a small amount of sugar, especially dextrose.[24] Acid is formed in small amounts by the pronounced color producers of orange or yellow, while the white organisms are often strong acid formers.

The coloring matter itself is a yellow lipochrome, insoluble in water, but readily soluble in alcohol;[20] the alcoholic solution having a golden-yellow color which passes over into an orange. Sulphuric, hydrochloric, or acetic acids change it to a bright yellow, and nitric acid will do the same, but the color will then fade entirely. The addition of a few drops of potassium hydroxide produces an orange red which then turns into a red. A drop of sulphuric acid introduced into the residue which remains after boiling, the color of this residue being of a reddish, golden-yellow, will give at first a blue-violet and then a blue-green shade. Potassium hydroxide gives a purple-red color but if thinned with alcohol the red color, noted above, shows.

Color production is very easily lost; Kolle and Otto[11] noted that heating a culture to 85° C. caused *Sta. aureus* to lose chromogenic power and become white and that it would also lose it through long cultivation on artificial media or by repeated inoculation into animals. Konradi[12] had noticed that this orange species would lose its chromogenic power if grown too long in distilled water or tap water but that it could recover it again if inoculated into animals. Winslow and Rogers[23] noticed a loss in the power of color production if the organism was kept at a temperature of 50 to 55°. Konradi's observations indicates that color production is associated with rich food of animal origin.

Identification by Color Production.—Inoculated into milk, the pathogenic species of these organisms grow readily and produce some acid from the lactose of the milk, although usually not enough to coagulate it unless the milk is heated. Some related forms afterwards digest the milk but *Sta. aurantiacus* does not. *Sta. aurantiacus* will not reduce nitrates to nitrites when potassium nitrate is added to the ordinary media, but the yellow form, *Sta. aureus*, is a nitrate reducer. *Sta. aurantiacus* is not a liquefier of gelatin; *Sta. aureus* is. These two characters seem to be fairly constant and may be used to distinguish the two organisms.[5] The color changes seem to be variable in cultures isolated from different sources, as shown in the following table, Winslow (1908):

Percentage of Cultures from Each Habitat

Color of pigment	Parasitic	Saprophytic
White	53	47
Yellow	28	72
Orange	76	24
Red	4	96

Winslow classified the members of this group on the basis of the color of the colonies but the shades are variable. Hucker[5] has supplemented the color classification by a comparison of the gelatin liquefaction, nitrate reduction, and the utilization of urea and ammonia. The use of serological tests was not successful with this group in establishing the relationships within it but the other tests seem to be sufficient to determine the main groups.

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CHAPTER XXIV

ACTINOMYCES CHROMOGENUS

(THE COLOR-PRODUCING THREAD ORGANISM)

(*Actinomyces scabies, aerugineus, intermedius, nigrificans, etc.*)

Nutrient agar exposed to the air frequently shows, after an incubation period of 1 week or 10 days at room temperature, small white colonies with a halo of brown tint for a distance of 1 to 2 millimeters from them (Fig. 107). The colonies are very small, rarely exceeding 2 to 3 millimeters in diameter, but the brown tint makes them very conspicuous on the plate. If the margin of such a colony is examined, it will appear as fine threads extending out into the medium; the ordinary bacterial colonies show the margin as a smooth line made up of little organisms which appear as granules. The surface of the Actinomyces colony may be smooth and waxy, or covered by a fine, ash-gray powder.

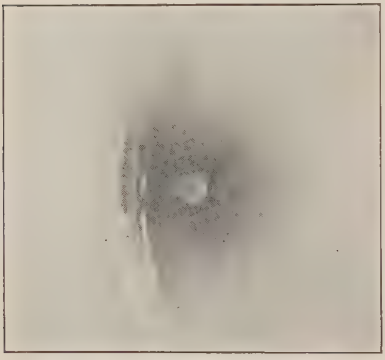

FIG. 107.—A colony of *Actinomyces chromogenus* showing dark coloration around colony on agar.

A transfer to another tube of agar shows, in 1 week or 10 days, the same dark-brown coloring matter diffused through the entire tube, but even more striking is the odor of newly turned garden soil which the organism has imparted to the air in the test tube. These organisms are so abundant in rich garden soil that they give it this odor and have been named by one investigator,[9] *Actinomyces odoriferus.*

The Organism.—Under the microscope, very fine branching threads, irregularly broken, appear, especially in stained preparations made by removing one of the colonies with a little agar to a clean cover glass. A little water is added and another cover glass squeezed against it with a firm, rotary pressure, so as to grind up the very leathery sphere. The two covers are then separated, air dried, fixed in the flame, and stained in an aqueous solution of gentian violet for 3 to 5 minutes; the stain is removed by washing under the tap; air dried and mounted in Canada balsam.

The branching mycelium seems to be made up of a tube which stains very faintly with cell contents of a dark-violet color. Irregular fragmentation occurs at any place in the mycelium, separating it into pieces, any one of which may reproduce the

organism. In addition to this fragmentation, occasional bead-like chains of conidia may be observed. These conidia are produced in essentially the same manner as the other fragments. The branches, which extend into the air, dry rapidly and the contents of the tubes contract into egg-shaped fragments which later may pull away from each other for some distance but still remain connected by the thin membrane

FIG. 108.—Ærial spores in various Actinomycetes: 1, conidia-like chains resembling those of the molds; 2, segmentation of mycelium to form spores; 3, chains of spores are corkscrew shaped; 4, method of origin of spores at tipe of hyphæ. (*After Drechsler.*)

(Fig. 109). The branches, which form the conidia or gonidia, often assume a spiral form as the result of the formation of the spores within them, as is shown (Fig. 108). The spiral twist may at times be sinistrorse (to the left) and at other times may be to the right, dextrorse. Drechsler[3] claims that as these spirals are constant their form may be used to determine the species.

Some of the colonies are dirty white in color and have a waxy appearance, but many on them have a fine white, or gray, growth, especially at the middle of the colony. This growth is the fructification of the aerial branches of the organism. The amount of fructification which is produced is variable, but a little can be seen in most cases.

The Coloring Matter.—The coloring matter produced is very soluble in water and diffuses out from the colony. It is variable in amount, being dependent to some extent on the presence of sugars in the medium. It is evidently a by-product of the nutritive activities of the organism and may be regarded as waste material. Beijerinck[1] identified it as chinon ($C_6H_4O_2$) derived from the protein or peptone in the media. It is only produced in the presence of an abundance of air.

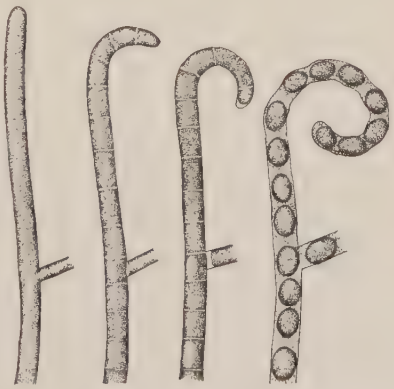

FIG. 109.—Origin of ærial spores in the Actinomycetes. The protoplasm cleaves internally into block-like segments which later round up as spores inside the old cell wall which collapses between the individual spore, diagrammatic. (*From Lieske.*)

Varieties, Strains and Species.—Somewhat similar colonies in size and general appearance do not produce color. These have been put into a general group, *A. albus*, by the older classifiers of bacteria, but they probably have as many strains as the *A. chromogenus* group.

This large and diverse group, *Actinomyces chromogenus*, has been broken up into numerous species by Krainsky[4] who attempted to separate them on the basis of their color production on various media and by their production of various enzymes. Waksman and Curtis,[11] following similar methods, added numerous new species from America. The reliability of physiological reactions is to be questioned as a basis for classification, and the morphological studies of Drechsler[3] probably represent a surer step in advance in the relationships of the strains which have been put together as *Actinomyces chromogenus* and *albus*. Morphological structures are usually more permanent than physiological reactions, although they also, may at times, be only culture reactions. The production or non-production of coloring matter is variable, according to all the authors who have studied the group, including Lieske[5] who monographed the European forms.

The futility of such classifications is admitted by Conn[2]: "It has proved impossible to identify with certainty any organism thus far studied (in New York) with any species described by Krainsky." While of Waksman and Curtis, he adds, "Their descriptions are even more incomplete than Krainsky's," but does not attempt to name the species which he found, contenting himself with numbering some of the more individual cultures.

The Life of the Actinomycetes in the Soil.—Münter[8] finds that the Actinomycetes, with which he worked, all took part in the destruction of organic substances to form ammonia. They make use, also, of the ammonium compounds; so that, in soil, the destruction of the nitrogen compounds by the action of Actinomycetes must be fairly complete. In fact, growth is best on some of the ammonium compounds. On the other hand, these organisms do not seem to fix nitrogen from the air and have a hindering effect on the activity of the Azotobacter species which fix free nitrogen in the soil. Their function would seem to be to destroy the organic nitrogenous material which finds its way into the soil, so they are especially abundant in soils in which there is considerable humus.

Munter's results have been confirmed by Waksman[12] who finds that when strains of the soil Actinomyces were grown in laboratory cultures on milk, blood, white of egg, gelatin, or blood serum, many of them were able, by the action of rennet-like and proteoclastic enzymes, to produce liquefaction and digestion. The various strains showed marked differences in ability to utilize these nitrogenous substances, but most of them could use some of them. Starch seemed to be the best carbohydrate medium, although all the common sugars, and also cellulose, could be utilized. The organisms are very resistant to drying out,[10] and may even remain alive for 2 years on filter paper in culture tubes without any source of carbohydrate. The filter paper fibers are partially dissolved at the end of that time, so that the Actinomycetes are able to utilize the cellulose as a source of energy and can live in the soil indefinitely on cellulose. Conn,[2] after observing that sod soil is especially rich in the Actinomycetes, containing 38 per cent of its flora of this type, as compared to 20 per cent on ordinary soil, has suggested that they are especially active in the destruction of the materials in the grass roots.

Actinomycetes as Disease Producers on the Underground Portions of Plants.—Some strains, especially those which produce the intense brown-black color, are semiparasitic on the potato tuber, stimulating the corky layers to the formation of an abnormal amount of irregular cork cells, the swollen, protruding portion being called a "scab." This disease is one of the commonest found on tubers grown in rich garden soil where this type of organism flourishes. Liming increases the activity of these organisms, as they seem to flourish in an alkaline soil.[6] A similar cork stimulation and formation occurs on the garden and sugar beet. These organisms have been named *A. scabies* and made a distinct species, but difficulty in naming Actinomyces from any descriptions already given was encountered by Millard and Burr[7] in the

species isolated in England from scabby potatoes. Eleven of the twenty-four strains isolated produced scabs of varying types but, with the exception of one species identified as *A. scabies*, these twenty-four strains did not agree with any of the published descriptions, and so were described as new. It is evident that in this group a great variation of characters occurs and investigators are still adding to the list of supposed new species.

Actinomycetes as Animal Parasites.—Species of Actinomycetes produce *actinomycosis*, or lumpy jaw in cattle, by the formation of large growth in the tissues of the lower jaw and tongue. Similar infections occur in other animals and over 100 cases of infection of man in America were known up to 1902.

The Position of the Group in Relation to Other Microorganisms.—The group of the Actinomycetes seems to occupy an intermediate position between bacteria and molds. The true bacteria may be only Actinomycetes that have broken up into fragments, have lost the thread-like form and have developed other physiological characters; or the thread-like species may be only rod-like forms which have, under cultural conditions, assumed the filamentous habit.

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CHAPTER XXV

BACILLUS RADICICOLA

(THE ORGANISM IN THE NODULES ON LEGUMINOUS PLANTS)

(*Bacterium radicicolum; Rhizobium radicicolum; and R. leguminosarum*)

The clovers have been known since the beginning of the nineteenth century to be soil enrichers while wheat and potatoes are soil impoverishers. It had been long known that the leguminous plants, to which the clovers belong, took very little nitrogen from the soil and that, while they

FIG. 110.—Roots with nodules of (*a*) young alfalfa, (*b*) young pea. (*From Löhnis and Fred's "Agricultural Bacteriology."*)

themselves were particularly high in nitrogen, they left the soil richer in nitrogen for a succeeding crop of wheat, rye, or oats. This superiority of the leguminous crops in restoring soil fertility seemed to lie in the peculiar growths found on the roots, known as *nodules* (Fig. 110).

The Organism.—The nodule organisms can be examined by crushing up a nodule between cover glasses and staining the contents, or, may be easily isolated into pure

cultures on ash agar (a medium composed of the soluble salts of 5 grams of hardwood ash in 1,000 cubic centimeters of water, 15 grams of agar and 10 grams of cane sugar). The appearance of the bacteria obtained from the nodules is very different from those in the cultures (Fig. 111).

The organisms in the nodules are irregular in shape and very vacuolate; those from the young cultures are short, motile rods surrounded by a layer of slime. The interest-

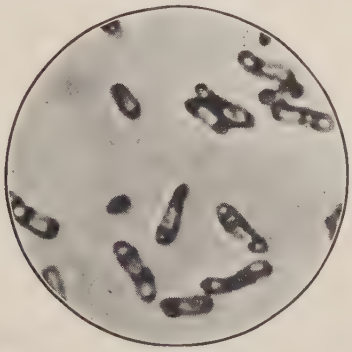

FIG. 111.—*B. radicicola* from alfalfa, bacteroid cells only. × 1,500. (*Edwards and Barlow.*)

ing fact must be noted that while the organism is actively working in the nodule, it assumes these peculiar forms, once considered by bacteriologists to be degeneration or involution types, while the more usual types found in the cultures were assumed to be the true form of the organism (Fig. 112). Fred[13] regards the bacteria in the nodules, however, not as degenerate or transformed organisms but as a step in the development of a life cycle; and Pinoy[23,2] has tried to connect them with the Myxobacteria, a

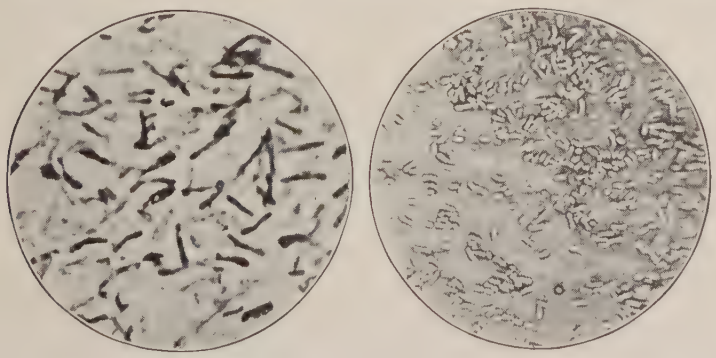

FIG. 112.—*B. radicicola* from alfalfa nodules: *A*, branching forms; *B*, small rod forms. × 1,000. (*Edwards and Barlow.*)

group in which, while a plasmodium is formed, the organisms, although they live colonially, do not lose their identity (see page 295). The nodule bacteria as they age in cultures, pass from one form into succeeding ones in a regular sequence.[1,17,29]

Earlier Accounts of the Nodules and Nodule Bacteria.—The first description of the nodules was by Malpighi in 1687, who considered them to be galls, and therefore, abnormalities. Woronin[32] made the first

observation that the cells of the nodules were filled with living bacteria. The nodules themselves were considered by Eriksson[11] and Cornu[10] to be simply metamorphosed side roots changed by the stimulus of the bacteria to form a fleshy growth of cells in which the bacteria lived. Frank[12] found that he could get no tubercles in sterilized soil and Ward[30] confirmed this observation with sterilized water cultures. Prazmowski[25] was evidently the first to see the entrance of the organisms into the root hairs of the plants (Fig. 113) and to use pure cultures from media for their infection.

Brunchorst had already observed[7] that the bacteria in the tissues were irregular in form and size and had called them bacteroids (bodies resembling bacteria) but Beijerinck,[2] after obtaining the organisms in pure cultures in artificial media, found that

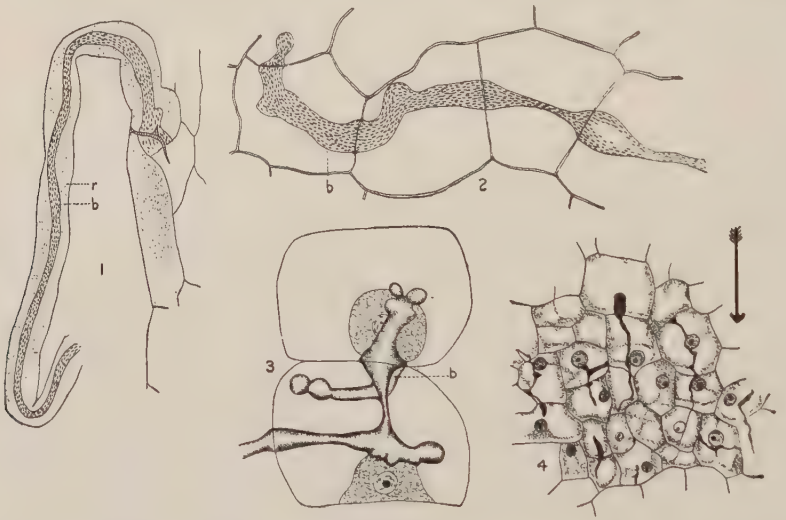

FIG. 113.—Bacteria of root nodules, *B. radicicola:* 1, an infected root hair; 2 and 3 bacteria imbedded in thread of slime penetrating from cell to cell; 4, infection strands (in black) all progressing in one direction. (1, *after Prazmowski;* 2, *after Dawson;* 3, *after Stefan;* 4, *after Peirce.*)

here, too, they had a tendency to lose the rod form and become irregular in outline. He had noticed that the different varieties of legumes had specialized types which would grow on one type of legume but not on many of the others, and arranged them, on the structure of the nodules themselves, into three groups: from Phaseolus, from Lupinus and from Robinia. The question of the number of strains which occur is still a disputed one which numerous cross inoculations have not entirely decided.

The life history of the nodule organisms would seem to be: (1) the invasion of the root hairs of the plant (Fig. 113) by a mass from the soil; (2) the formation of long strands composed of the bacteria, probably imbedded in slime, advancing, as an army, through the cells and the cell walls, this slime being formed from some of the products of the cellulose which the bacteria have dissolved or from their own walls; (3) the arrang-

ing of the bacteria on the cells of the nodules as a slimy layer surrounding a large central vacuole, with the cell nucleus crowded off to one side; (4) the transformation of the rod-shaped organisms into the bacteroids.

The Infection of the Plant.—The bacillus enters the plant through the root hairs but, as soon as a root hair is infected by a strand of organisms, it bends sharply into the form of a shepherd's crook and cannot be infected again. There seems to be no evidence that the organism is attracted to the root hair by a chemotactic stimulus. The meeting of the two apparently is a chance one, so that the soil must contain a large number of the organisms if a sufficient number of the root hairs are to be infected. Peirce[22] estimates that on bur clover only one root hair in a thousand is invaded by the bacteria.

The walls of root hairs being composed of cellulose are readily soluble and Fred[14] found the bacteria in little clumps pressed against their sides. Under these masses, the cell walls soften, the bacteria press into the cell, and start multiplying immediately.

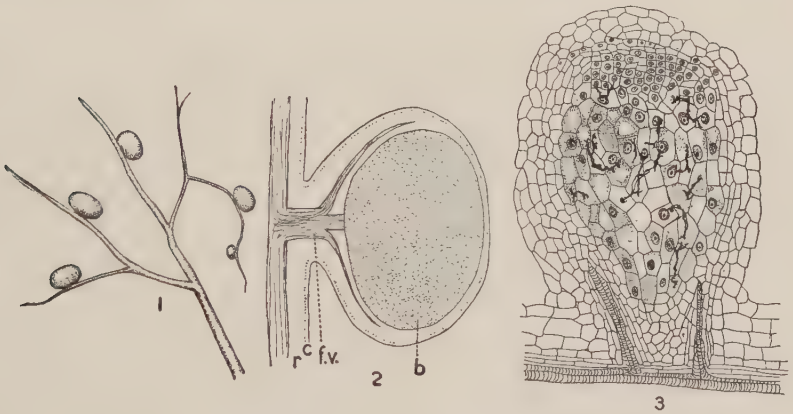

FIG. 114.—Structure of root nodules on common bean (*Phaseolus*) and horse bean (*Vicia faba*): 1, nodules on root, natural size; 2, diagram of longitudinal section of root and nodule of Phaseolus, × 10; *r*, root, *c*, cortex; *f. v.*, fibrovascular bundles; *b*, bacteria-infected mass of tissue; 3, section of young nodule bacterial strands in black. (*After Spratt.*)

This infection tube is not sharply defined, being a zooglea of imbedded bacteria usually located at the center of the cell.

Bacillus radicicola in cultures often produces slime in quantity, saccharose agar being apparently especially adapted to its formation.[8] A 2 per cent solution of saccharose, sterilized, and inoculated with the nodule organism will become very gelatinous at the end of 2 weeks. This gum is closely related to the dextrins and no trace of nitrogen in any form can be detected in it, so it would seem to have very little connection with the nitrogen assimilation process. The gum seems to arise from the outmost portion of the bacillus wall itself. The organisms undoubtedly produce a similar gum in the cells of the host plant and the invasion strands are composed of bacteria imbedded in slime.

In order to produce tubercles, the organisms must be in contact with the roots; if they are inoculated into other parts of the plant, such as the aerial portions, they will die off in 3 to 4 weeks without any effect on the host itself or any marked increase in numbers.[33] The bacteria seem to find, in the cells of the roots, conditions which

are not duplicated in the cells of the stems and leaves, and they are unable to migrate through the plant to their normal habitat.

After the death of the leguminous plants, the nodules go to pieces very rapidly, the cells becoming filled with a milky liquid in which are to be found the empty and unstainable cell walls of the organisms, the bacterial cell contents having apparently escaped into the liquid.

Are the Nodules Modified Roots?—Peirce[22] claims that, in bur clover infection, the nodules are formed from the same tissue as that which gives rise to the lateral roots, so that the nodules might be regarded as very highly modified lateral roots. Miss Spratt[27] investigated nodules from a variety of legumes and is certain that they are not side roots arising, as such roots always do, from the vascular region, but that they come from the outer portion of the root, *the cortex*, and are exogenous in origin. They are composed of a mass of parenchymous tissue which is supplied with a varying number of vascular strands; in Phaseolus, the basal vascular strand divides into four; in Vicia into two; and in Trifolium the single strand remains undivided for some time (Fig. 114).

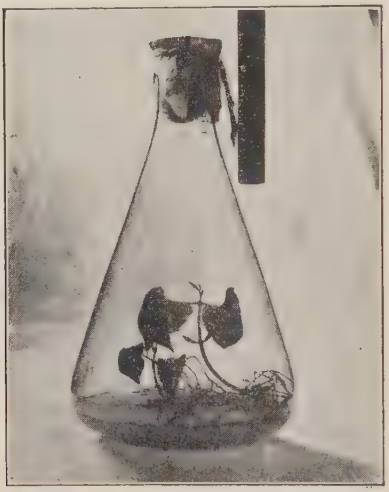

Fig. 115.—Garden bean growing on ash-maltose agar in 1½-liter flask. (*Edwards and Barlow.*)

The Production of Nodules in Pure Cultures.—The lack of nodules on leguminous plants grown in sterilized soil has already been noted. Vetch or bean plants may be grown from sterile seeds on agar containing maltose and the water from hard-wood ashes, and inoculated (or left uninoculated) with pure cultures of the *B. radicicola* (Fig. 115). The agar being transparent, the whole process of the growth of the nodules can be followed.[18] These pure culture methods eliminate, also, all danger of contamination with other bacteria, although few other organisms present would grow well on the agar. Sterile seeds and the pure nodule cultures induce nodule formation.

THE SYMBIOSIS OF BACTERIA AND LEGUMINOUS PLANTS

The physiological relation of the nodules and the legume plant upon which they are produced is one of the most interesting examples of symbiosis (sym = with, bios = life; a living with). The legume forms carbohydrates and absorbs the water necessary not only for itself but also for the bacteria; while the bacteria use part of the energy derived from the carbohydrates for the fixation of the nitrogen of the air to form their proteins. The presence of the bacteria makes it less necessary for the legume to absorb nitrates, since some of the nitrogen compounds are undoubtedly formed by the organisms in excess of their needs, as Klein[19] was able to demonstrate nitrates in abundance in the cell sap in the tubercles of Phaseolus. Much of the nitrogen which they form is bound up for a time in the bacterial protoplasm and only released with their death and the disintegration of the nodule, but there seems to be such an excess of it that the legumes themselves are richer in nitrogen with nodules than without them. Much of this nitrogen passes up into the growing seed and the nodules lose some of the element, as Stoklasa has shown in the following table:

Nitrogen Content Dry Weight	At Blossoming Time	At Time of Setting of Fruit	After the Ripening of the Fruit
Root nodules	5.2	2.6	1.7
Nodule free roots	1.6	1.8	1.4

This accumulation of nitrogen in the tops of the plants as the seeds ripen is illustrated by the diagram from Whiting[31] (Fig. 116).

The symbiotic relationship is not a voluntary one at the beginning of the combination, since the bacteria force themselves into the root hairs in exactly the same manner as does a pronounced parasite. The plant is unable to resist them, however, so they establish themselves in the legume cells, but should it be that it is a bacterial variety that is not especially adapted, the host plant may get the upper hand at once and destroy its invaders. After the legume plant has become stronger than the bacteria, by the absorption of nitrogenous compounds from them, it is able to maintain

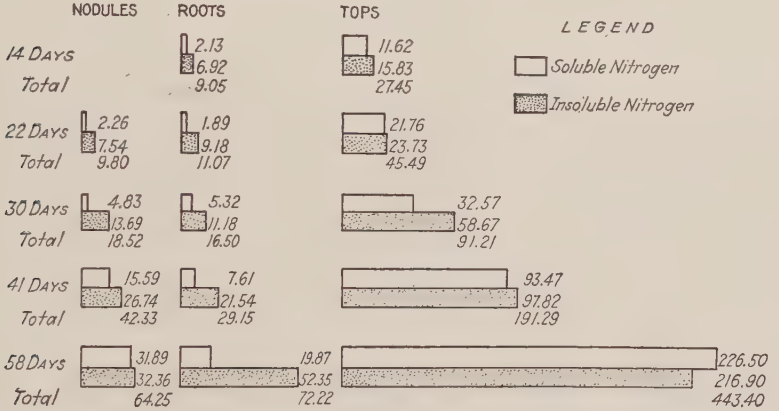

FIG. 116.—The nitrogen in various parts of cowpeas at various stages of growth; in milligrams per jar of five plants. (*From data by Whiting.*)

itself and to establish a real partnership. In the later stages of the partnership, the plant draws very heavily on the substance of the bacteroids and may actually resorb some of their proteins. At this time, the legume turns the tables and is really able to use the bodies of these bacteroids as reserves of nitrogen ready for absorption.

Hiltner first suggested that there are six gradations by which the immunity of the plant is exhibited:[18a]

1. The organisms cannot enter the plant at all because of its resistance.
2. The organisms can enter the plant, but after they get in they are absorbed by the plant and are never able to form nodules.
3. The organisms enter the plant, produce nodules, but fix no nitrogen.
4. The organisms enter the plant, produce nodules, fix nitrogen; the nitrogen is absorbed by the host plant. This is real symbiosis.
5. The organisms enter the plant and are so efficient in obtaining food that they injure the plant to some extent.
6. The organisms enter the plant but are so destructive that they become pure parasites and destroy it.

We have in this series suggested by Hiltner all the gradations between an organism which is helpful and one which is really injurious and dangerous to the life of the

plant into which it finds its way, in other words, between symbiosis and parasitism. Fuhrmann[16] has suggested that the stage when bacteria in the nodules stain brown-red instead of blue in an iodine solution marks the beginning of the fixation of atmospheric nitrogen. The nitrogen compounds of the host plant have become exhausted and the bacteria are forced to resort to other means to get supplies of nitrogen.

Do the Bacteria Fix the Nitrogen or Do They Only Serve as a Stimulus?—Beijerinck[3] has come to the conclusion that the organisms in the nodules fix nitrogen but that the protoplasm of the host may be a necessary catalyst. Indirectly, of course, the organisms probably serve as the stimulus to such fixation but in themselves they do not alone fix much nitrogen. The nodules on some of the large woody or shrubby legumes are not numerous, although it is known that these plants fix large amounts of nitrogen and Beijerinck hints that, perhaps, the plant itself does the assimilation. Legume bacteria in cultures or when growing free in the soil fix only small amounts of nitrogen.[13,15] If the free organisms can fix nitrogen, they should in culture media lacking that element, but there is no experimental evidence that they are able to do so in any quantity. The accumulation of the products of fixation in the bacterial cells has been suggested as the cause of such small fixation in cultures or soil; in the latter cases, the nitrogen compounds increase in the solution surrounding the organisms and stop their activity.

Nitrogen Fixation in Similar Nodules on Other Plants.—Similar nodules occur on various other plants which are not legumes, among which may be named Alnus, Ceanothus, *Myrica Gale* and Eleagnus. Some investigators, such as Bottomley[4] on *Myrica Gale* and Miss Spratt[26] on Eleagnus, claim to find organisms in these outgrowths identical with those of the legume nodules and capable of fixing nitrogen in cultures. Sometimes these organisms are associated with certain blue-green algæ on the roots of these plants, the algæ furnishing the carbohydrates and the bacteria fixing the nitrogen of the air to make nitrates.[5] Nitrogen fixation by these organisms and their relationship to *B. radicicola* is still questioned by other investigators.

The nodules of the non-legumes, such as Ceanothus, Cycas, Alnus, and Myrica, are structurally different from those of the legumes, being modified side roots while those of the legumes are a mass of parenchymous tissue occupied by the bacteria and a few strands of conducting tissue.[26]

Growing B. Radicicola on Non-leguminous Plants.—The possibility of growing legume organisms upon cultivated plants, such as corn, has induced investigators to study infection with root bacteria on other plants than the *Leguminosæ*. The advantages in having a plant produce its own nitrates from the air is obvious; the addition of nitrates to fertilizers would be unnecessary and, even if the crop did not enrich the soil, it would not impoverish it of its nitrogen. All infections with nitrogen-fixing root bacteria have failed on cultivated plants[9] and no nodules have been seen or developed on any of them except the legumes.

A very elaborate attempt to induce the nodule-forming bacteria to grow on non-leguminous plants made by Blunck[6] was unsuccessful. He tried to adapt the legume bacteria to other plants by growing them on boiled root extract made from the plant to which he wished to accustom them, or, on gelatin containing the root extract. After the bacteria had apparently adapted themselves to the root extract, they were grown on boiled roots. The antibodies of the living root presumably would be active and make it impossible for the organisms to grow on live roots; as the next step, a

little extract from living roots was ground up, passed through a Berkefeld filter and added to the medium. When it was felt that the cultures ought to be thoroughly acclimated, they were placed on the roots of living plants, put into a receptive condition by starvation; but with unsuccessful results, no nodules being formed.

Soil and Seed Inoculation with Legume Bacteria.—The original method of inoculating a field, on which clover was to be grown, with soil from some other field where the clover was successful, as was formerly done in the Netherlands, has given way to the use of pure cultures of the particular organisms desired. The original pure-culture method was to dry the organisms on masses of cotton, but as desiccation proved fatal in a percentage of the cases, it was abandoned. At present, the bacteria sent out by the U. S. Department of Agriculture are grown on an agar jelly in which there are the soluble salts of wood ash and a little sugar, or in liquid cultures. The culture is mixed with the seed to be inoculated, so that every seed receives some organisms. The ease and cheapness of this method have made it more popular than soil inoculation where 200 to 500 pounds of soil must be scattered over each acre in order to attain the same results.[21]

In order to inoculate *any* legume at least seven different cultures are necessary since the nodule bacteria of one legume will not necessarily cause nodules on another legume of a different species or genus. This fact has been determined by cross-inoculations and the following seven groups on cultivated legumes established:

1. Alfalfa bacteria. Also bitter clover, button clover, California bur clover, Fenugreek, Southern bur clover, white sweet clover, yellow sweet clover, yellow trefoil.

2. Red-clover bacteria. Also alsike clover, crimson clover, hop clover, low clover, mammoth red clover, rabbit-foot clover, white clover.

3. Vetch bacteria. Also Canada field pea, garden pea, hairy vetch, broad bean (horse bean), lentil, narrow-leaf vetch, purple vetch, sweet pea.

4. Garden and navy-bean bacteria, including all the common garden and field varieties of beans.

5. Lupine bacteria. Also serradella, sundial (wild) lupine, European yellow lupine.

6. Cowpea bacteria. Also Florida beggarweed, jack bean, Japan clover, Kudzu, Lima bean, partridge pea, peanut, pigeon pea, tick trefoil, tepary bean, Deering velvet bean.

7. Soybean bacteria.

These seven strains would inoculate any of the cultivated varieties of the legumes but at least five other strains on wild species are known:

8. Hog peanut.

9. Lead plant.

10. Trailing wild bean.

11. Black or common locust.

12. Wood's clover.

The reasons for the physiological difference in the races of legume bacteria are not known, but that it is not purely physiological is shown by the arrangement of the flagella around the organisms.

Some nodule bacteria have flagella all around them, others, only at one end. An investigation of this distribution of flagella by Löhnis and Hansen[20] convinced them that the strains derived from the nodules on the roots of clover, sweet clover, alfalfa, vetch, pea, navy bean, lupine, black locust, Amorpha and Strophostyles were peritrichous (all over the bacteria) and the strains from cow pea, soybean, peanut, beggar weed, Acacia, Genista, and Cassia were monotrichous (at only one end). The physiological characteristics of the organisms of the two groups further confirmed this division.

Conditions of Successful Inoculation.—After the cultures are diluted and applied to the seed, it must be sowed as soon as possible.[15] The soil must be in good condition; not too wet; and with a neutral or alkaline reaction.

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CHAPTER XXVI

TWO PLANT PATHOGENES

BACILLUS CAROTOVORUS (THE ORGANISM OF THE SOFT ROT OF VEGETABLES)

[*Erwinia carotovora* (Jones) Holland]

Some vegetables have such acid juices that the bacteria cannot thrive in them, but other plants are sufficiently neutral in reaction that bacteria grow readily. Cabbages, cauliflowers, parsnips, carrots, turnips, and some bulbs, such as that of the hyacinth, rot from the attack of bacteria. The tissue of the plant becomes soft and pulpy with a water-soaked appearance, and, if a bit of it is placed in water and examined under the microscope, it will be found to be alive with great numbers of short, motile bacilli.

The isolation of this organism is very easy on ordinary nutrient agar. The large white or cream-colored colonies develop quickly at room temperatures, so that they may be picked off in 2 days. This organism was first isolated by Jones[4] who, as he found them in rotting carrots, gave it the name, *Bacillus carotovorus.*

The Organism.—Stained with the special flagella stain, it is a short rod with rounded ends and 2 to 5 flagella arranged around the body (Fig. 117). The size is 0.7 to 0.8μ by 1.5 to 5.0μ. No spores are visible and none seem to be formed in any stage of development.

FIG. 117.—*B. carotovorus*, stained for flagella. (*Jones.*)

The Organism in Cultures.—Gelatin cultures are rapidly liquefied and nitrates reduced to nitrites. A small amount of gas is produced in sugar broths (only a few bubbles) and the production seemed to be somewhat erratic in its appearance. The gas production is early in the growth of the organisms and is always accompanied by the production of enough acid to redden litmus milk.

Inoculations with pure cultures into cabbage or carrots produce a soft rot similar to that observed in the specimens from which the cultures were isolated. The inoculations must be made as a puncture, which suggests that, in nature, the entrance of the organism into the host must be from injury which permits it to enter the interior of the fleshy root or leaf. Once within the host plant, it spreads through the intercellular spaces and dissolves out the middle lamellæ, allowing the cell walls to slip on each other. The cells, without the cement which holds them together, fall apart very readily and show the symptoms of what we term *soft rot.*

The Enzymes.—The enzyme which this organism secretes has been studied in detail. The middle amella-dissolvinge nzyme, *pectinase*, can be isolated in such a pure form that slices of carrot placed in it soften in a few days with no bacteria present. The softening does not extend into the interior of the slice, since the enzyme penetrates

with difficulty to any depth. The bacteria must carry enzymes with them as they find their way through the intercellular spaces, the action slightly preceding them and opening the way for their advance.[5,6]

The organism causes rot only when freshly isolated from some of the rotting vegetables and, if grown in cultures for some time, this ability is entirely lost, and it becomes a true saprophyte. The pathogenicity is very weak and laboratory cultivation so rapidly destroys the ability of the bacillus to rot vegetables that it may be classed as a *facultative parasite.*

Variation in the Physiological Reactions of the Organism.—The organism shows the greatest variability in its physiological reactions. Jones, Harding, and Morse[5] isolated 37 cultures from a variety of sources and found gas production with the sugars to be highly variable; some strains producing a little gas with lactose; others a little with glucose; some with mannit; some with all of them; and some with none of them. Jones,[4] in his original account of the organism, found about 5 per cent of the closed arm of a fermentation tube would be filled with gas if dextrose, sucrose, lactose,

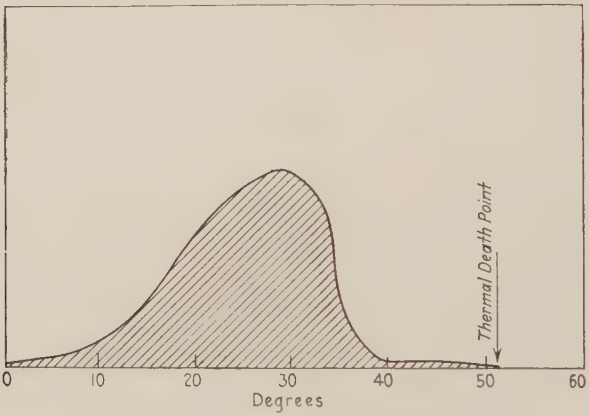

FIG. 118.—The growth curve of *B. carotovorus.* (*Jones.*)

or mannit were used, but none with glycerine. This gas had the composition of 20 per cent of CO_2 and 80 per cent of explosive gas, presumably hydrogen. These proportions suggest, at once, the results obtained with *B. coli*, and Jones tried cultures of this organism for comparison, evidently suspecting that he had only a strain of *B. coli* which was a weak gas producer. The morphology of the organisms is not markedly different, so it may be safely assumed that the soft-rot bacillus is one of the members of the very diverse *B. coli* group which, at times, has acquired the faculty of becoming parasitic on vegetables. Strains of *B. coli* are known to produce bud rot of the cocoanut in the tropics, although strain of animal origin live only a short time (200 days) in the soil.[11]

The soft-rot organism is evidently a soil type with its best growth about 25 to 30°C. (*B. coli* thrives at a somewhat higher temperature), a minimum about 2°C. on gelatin, and a maximum temperature of 38 to 39°C. (Fig. 118).

The plant pathogenes must show considerable range in their tolerance to acids, since some of the host plants have a relatively high acid content, especially of the organic acids. *B. carotovorus*, for example, will grow with a pH 4.8 content of sulphuric, citric, malic, and tartaric acids. Some acids, such as acetic, seem to have toxic, in addition to the acid effects, which they produce.[14]

BACILLUS AMYLOVORUS (THE ORGANISM OF FIRE BLIGHT OF PEARS AND APPLES)

[*Erwinia amylovora* (Burrill) Committee S. A. B.]

The death of the young growing twigs of the pear tree has been noted in the United States from the time of the earliest settlement. A twig or small branch after putting out leaves, or flowering normally, suddenly wilts, turns brown, and dies. If the bark of the portion to which the dead twigs are attached is examined, it, also, will be found to be dead and shriveled to a point just a little beyond the brown leaves. A narrow band of water-soaked tissue is apparently the meeting place of the healthy and dead portions, the branch seeming to die from the destruction of its cambium layer. The advance of dead bark during the season involves not only the twig or small branch, but also the larger one from which it arises. During the winter the progress of the disease is checked, to be renewed with the warm rains of the following spring when the death of the cambium of the tree continues until large limbs are encircled and die, or are partially killed, producing cankers of dead tissues readily invaded by other organisms, especially fungi. It is believed, today, by many investigators of plant diseases[9] that many, if not practically all, of the cankers on apple and pear trees are the result of an infection at some time by fire-blight organisms. Young apple trees are especially susceptible so that it is a disease that has to be feared in young apple orchards.

Location of the Active Organisms.—The organism producing the fire blight may be found in abundance in the region just in advance of the dead area. The intercellular spaces of this water-soaked area are full of organisms, and if a little piece of bark is placed in water, a cloudy milkiness soon appears, due to the bacteria escaping from the infected tissues; but, if the canker is old, or is not in a state of activity, the bacteria are scarce. Isolation on nutrient agar is comparatively easy.

The Organism.—The organism is a short rod with rounded ends, 0.6 × 1.5μ in size. The flagella are distributed all around the organism but mostly attached near the poles, according to Smith;[12] but Rosen[10] has found blight organisms (which will produce the typical lesions by inoculation) which have only a single polar flagellum. This discovery may mean that more than one organism may cause the same disease or that the staining methods adopted by previous investigators were faulty and often showed too many flagella. Rosen is inclined to adopt the latter view, since a strict interpretation of the theory of disease-producing bacteria implies that only a single species could induce a single disease reaction.

This organism is interesting because it is the cause of the first plant disease to be ascribed to the activities of bacteria. It was first isolated and described by Burrill[2] and his work was confirmed by Arthur in 1885. The voluminous literature on it has been well summed up by Heald[7] and by Smith.[12]

Artificial Inoculations.—The area around artificial inoculations appears water soaked, due to the air in the intercellular spaces of the cortex (occasionally that of the cambium) being displaced by fluid drawn from the cells themselves. In these cavities the bacteria swarm in numbers, and only occasionally occur inside the cells.[1] The

organisms seem to die off in the fall, although some survive as "holdovers" to start the infection in the following spring.

FIG. 119.—Development and life cycle of *B. amylovorus:* 1, large vegetative organisms imbedded in slime, forming a cavity (*schizogenous*) between cells by splitting cell walls; 2, slime strand inside host cell with clumping of the organisms to form central mass; 3, slime strand has invaded two cells, dissolved out cross-wall and cell contents to form cavity (*lysigenous*), the organisms are smaller; 4, the small organisms have formed a mass of material, surrounded by a wall, a *cyst*, to remain in a dormant condition. (*After Nixon.*)

B. amylovorus migrates through the tissue of the apple in the form of a dense column of individuals, held together by slime to form a zoogloea (similar to that of

B. radicicola), making its way as far as possible in the intercellular spaces, but spreading out from massed invasion into the cells themselves.[8] The vegetative life of the organism seems to be passed in this zoogloeal condition, but, in the winter, it passes over into small masses, *cysts*, which contain the slightly shortened individuals enclosed in a gelatinous envelope. In this resting condition it passes the winter (Fig. 119, 2, 4).

The injury to the apple tissue is the result of the formation of cavities formed between the cells (Fig. 119, 1) or by the dissolution of the cell walls so that several cells are thrown together (Fig. 119, 3). The former type of cavity, *schizogenous*, is the more common, especially in the early stages after infection, but as the advancing column of organisms arrives at the older tissue, they seem to invade the cells and dissolve the walls and contents to form *lysigenous* cavities.

The cells surrounding these schizogenous cavities seem to be poisoned or irritated in such a manner that they excrete part of their cell sap into the intercellular spaces. This liquid material is not poisonous to the plant itself, as has been determined by inoculating some of it into the tissue. *B. amylovorus* does not secrete a toxin as do some of the bacteria which produce animal diseases.

The rate at which the organism travels has not been determined but within 48 hours the water-soaked area containing them has been found to have extended ½ inch from the point of inoculation.

The disease is spread from tree to tree by means of insects, as was demonstrated by Waite.[13] The pedicels of the young fruit may show drops of sweet exudate in which the organisms are very abundant; bees or other insects visiting the flowers, may get them on their hairs or feet and carry them to other plants.

Prevention.—The prevention of the disease is limited to exterminating the organisms by removing the infected portions of the plant. In order to insure that all are removed, the cut must be disinfected with some strong germicide, such as a mixture of mercuric chloride and cyanide. The tool with which the twig is removed must also be disinfected in the same mixture, and the wound painted with ordinary paint in order to prevent the entrance of other fungus parasites which might find their way into the interior of the tree and destroy it entirely. The solution recommended[3] for disinfection is made as follows:

Mercuric chloride		1 ounce
Mercuric cyanide		1 ounce
Glycerine	3 parts	3½ to 4 gallons
Water	1 part	

The glycerine increases the penetration of the disinfectant and delays its rapidity in drying.

General Principles of Pathogenicity of Bacteria in Plant Tissue.—Three organisms have been discussed which induce pathological conditions in plants:

1. *Actinomyces chromogenus* strains, by their presence and secretions stimulate the formation of an unusual amount of cork on the surface of potato tubers. The organism produces substances which are *stimulants to growth.*

2. *Bacillus amylovorus* and *B. carotovorus*, by their presence and excretions poison the tissues and cause their death. The advance of these organisms is similar to that of an army in which the individual plays only a small rôle; it is an example of mass action. The life of these

microorganisms is largely intercellular; the advance between the cells is by means of pectose-dissolving enzymes which split the cells apart, *B. amylovorus* invades the host cells during the later stages of growth by cellulose-destroying enzymes, and absorbs the contents by proteoclastic enzymes. No specific toxin can be isolated which will, alone, produce these effects, but lack of results with inoculations of bacterial products may be the result of slow circulation of liquids in plants, of a weaker type of toxin or of a higher degree of resistance of plant cells. The chemicals formed by the bacteria are *poisonous or toxic to the host cells with which they come in contact.*

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CHAPTER XXVII

SOME TYPICAL ORGANISMS PATHOGENIC IN MAN AND THE ANIMALS

BACILLUS ANTHRACIS (THE ANTHRAX BACILLUS)

Introduction.—Pathogenic bacteria are less easily grown than the saprophytic varieties; they are much rarer and may require special methods. The relative scarcity of pathogenic species and the much greater importance of the saprophytic types under natural conditions are not generally recognized. The saprophytic varieties are the commonest inhabitants of soil, air and water, but, occasionally, an isolated organism, or group of organisms, occurs, which, if it falls on the particular region of the animal or human body to which it is adapted, will grow and produce a morbid condition.

The majority of laboratories where the pathogenic bacteria are studied depend on stock cultures already isolated. Fresh isolations are made only occasionally when fresh material containing the bacteria can be obtained. Some pathogenic organisms can be found in abundance in objects sent in for examination to city and state board of health laboratories, but other varieties are only rarely available. Many pathogenic forms are difficult to isolate and to grow with any certainty; special media are required and the discarded cultures must be carefully sterilized or disinfected. More than usual precautions must be taken that cultures are labelled properly, that the labels are legible and not easily erased, and care must be taken that the platinum needles used in making transfers are always properly sterilized before bringing them into contact with any other object.

The fascination of working with organisms which cause disease has focussed the attention of bacteriologists on this type since they were discovered about 50 years ago. The study has naturally fallen largely into the hands of the medical profession, or men who have been trained for the medical profession. In fact, many bacteriologists date the beginnings of this science from the discovery by Koch that pure cultures of bacteria could produce disease. The demonstration by Robert Koch in 1876 of the causal relation between anthrax and a specific bacillus marks the beginning of modern bacteriology.

This discovery undoubtedly led to the intensive study and accurate methods which have been carried over into other fields. The pathogenic types are of interest, however, to every one interested in biology since

they represent a peculiar mode of life and nutrition, and the study of a few typical disease-producing species should be a part of every course in general microbiology. In no other way can an understanding be acquired of those small but important enemies, the pathogenic bacteria.

Anthrax.—In domestic animals, "splenic fever," and in man, "malignant pustule," "wool-sorter's," or "rag-picker's disease" has been known for hundreds of years. An outbreak in Southern Europe in 1613 among cattle spread to the people and became such an epidemic that 60,000 persons died before it was stopped.[40] All warm-blooded animals are susceptible to the organism, it being most serious among cattle and sheep, and even attacking wild animals, such as deer.

As one of the popular names (splenic fever) indicates, animals which contract the disease develop a fever and the spleen

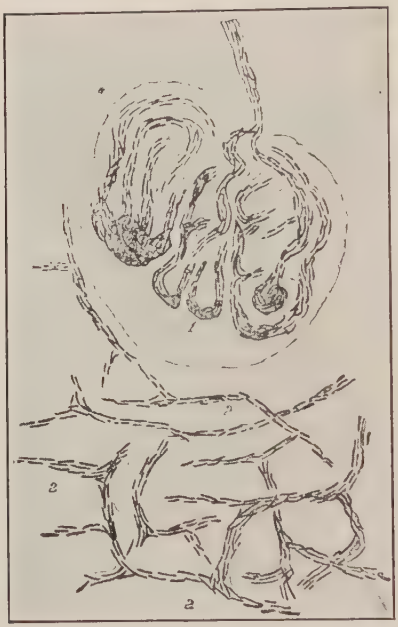

FIG. 120*a*.—Section through the kidney of a rabbit dead of anthrax with the capillaries filled with bacilli: 1, glomerulus; 2, capillaries.

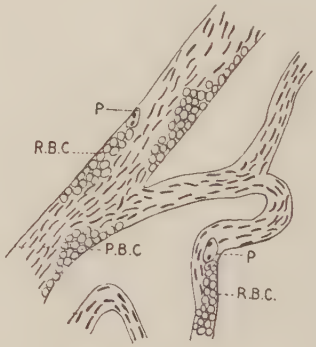

FIG. 120*b*.—Anthrax organisms in smaller capillaries; *R.B.C.* red blood corpuscles; *P.* phagocytes. (*After Martel.*)

enlarges. The blood becomes very dark red in color (almost black at times) and swellings appear under the skin. In man, the disease may show itself as a swelling or carbuncle on the skin where the infection has resulted from the handling of the carcasses or skins of infected animals, or it may appear as a respiratory disease among workmen handling the skins of infected animals, or the wool from sheep which have died of the disease, or rags which have been contaminated from the soil.

Infections due to the use of shaving brushes made from horse hair which came from animals which had died of the disease was one of the incidents of the World War.[30] The horse hair had been imported from China and had not been properly disinfected. In shaving, small abrasions were opened in the skin and into them the organisms from the hair found their way.

Infections in wounds is not necessarily fatal, but infection through the lungs or intestines is very likely to result in death. The method of infection in animals is not known in all cases but occurs usually in the animals grazing over land which has been

previously contaminated in some manner. The organisms enter the body from the grass or hay in the digestive tract and rapidly find their way into the blood stream where they grow with great rapidity and soon begin to check its flow, especially in the liver and spleen, owing to the formation of chains which catch and hold the corpuscles (Fig. 120).

Infections are especially numerous on land subject to overflow at some time during the year, especially if the soil is a black, heavy loam or a heavy, peaty soil. The organism seems to survive unusually well in such situations, probably in a resting condition or, possibly, actively growing as a saprophyte. The land along streams below woolen mills where the washings find their way into the water or tanneries where the refuse is poured into streams are breeding places for the disease. One of the badly infected spots in the United States is the land along the Delaware and Susquehanna rivers where the morocco leather industry brings hides from all over the world, but particularly from the Near East, Thibet, and China. Hides from these regions

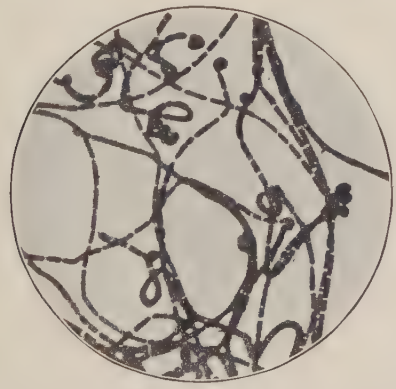

FIG. 121.—*Bacillus anthracis*, without spores in the cells. (*From Kral Institute.*)

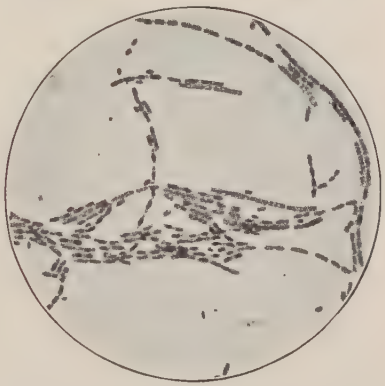

FIG. 122.—*Bacillus anthracis*, with a spore in each cell. (*From Kral Institute.*)

seem to be more often infected, the hides being removed and sold regardless of the danger to all workers who are compelled, subsequently, to handle them.

The Organism.—The organism, always found in the blood of infected animals and in such numbers as to stop the flow through the smaller capillaries, is a slender rod about 1μ in breadth and 5 to 10μ in length (Fig. 121). It is non-motile, forming fairly long chains, with cells marked by their sharp cornered ends. Spores (0.8 by 1.5μ) (Fig. 122) are readily formed in almost all cultures, if exposed to the air, although *asporogenous* strains are known. The spores are of special importance because of their great resistance to desiccation, aging, and disinfectants. They remain viable for years, under natural conditions, in the soil.

THE ANTHRAX ORGANISM AND THE GERM THEORY OF DISEASE

The Production of the Disease by the Organism.—The controversy which arose over this organism is part of the development of the theory of the causal relation of bacteria to disease; the steps leading to this conclusion are interesting to follow. Koch and Pasteur are given the credit for establishing and demonstrating the germ theory of disease, especially Koch, who carried the work through to a successful end. They builded on the labors of other men, however, and the history of this

theory shows how such fundamental discoveries await the man and the hour. To Koch fell the honor of fashioning and putting in place the keystone of the arch, but other hands cut the stones that held it in place.

The fact that this organism occurred in animals sick with this disease has been known since 1849.[3] At that date Pollender, investigating the blood of animals sick with anthrax, found countless numbers of motionless little rods. His results were confirmed by Brauell, in 1857, in the blood of men, sheep, and horses dead from the disease. The question turned on the relation of these rod-shaped organisms to the disease itself. Brauell kept blood infected with the organisms through four generations, for 4 to 5 days, and still found that it would produce the disease, but that if kept for 15 days it lost its pathogenic property. The little rod-shaped bodies appear in the blood 1 to 3 hours before the death of the animal and, occasionally, 8 to 10 hours. None of these rod-shaped bodies occur in the blood of animals which are not affected by the disease. They seemed to appear in the blood as a result of the pathological conditions of the plasma and were crystals or platelets, but were not alive. This viewpoint was upheld by Rudolph Virchow (Fig. 123), the founder of pathology. A pathologist at that time studied abnormal condition in cells and tissues without reference to the cause of those conditions.

FIG. 123.—Rudolph Virchow (1821–1902). Founder of modern animal and human pathology. Disease is due to an abnormal condition of the cells; the cause is to be sought in some agency which has induced this condition.

Virchow's opinion at this time was in keeping with his experience and training, and he came later to a quite different viewpoint when the effect of bacteria had been demonstrated. He was interested at this time in diseased cells and tissues; much less so in the organisms which often induce these changes from the normal.

"To Virchow we owe our conception of disease. Disease is not an entity entering the body from without. Disease is life, life which deviates from the normal. The causal factor may reside within or may come from without in the form of trauma, infection, intoxication, or what not, but the cause is not the disease. The disease is the abnormal life of the body cells. The methods of modern medicine are, therefore, broadly biologic, and along this road of promise Rudolf Virchow pointed the way."[41]

During this decade of the 'sixties Virchow was still interested only in the abnormal conditions and was not yet convinced that bacteria could poison the cells and bring about pathological changes.

Davaine.—Davaine[18] began at this time to study anthrax in France and maintained that blood without the organisms was incapable of producing the disease. The appearance of these bodies marked the beginning of the well-marked symptoms of the disease and their disappearance marked the cure of the animal. The number of these bodies in a drop of blood was estimated by Davaine[19] to be 8 to 10,000,000. Dried blood was only contagious for 24 hours and then lost its contagious properties

completely. The blood of a mammal mother might be filled with the organisms while the fœtus in the body was entirely free from them.

Davaine's views were combated with vigor in France; Sanson claimed that diseases other than anthrax could be produced by the same, or very similar, organisms, and that often animals, dead of a disease which seemed to be anthrax, showed no organisms in the blood. A French Commission appointed to study the matter found that the anthrax was contagious from animals in which no trace of an organism was to be found, and their presence was not at all constant in the blood of the diseased animals. Inoculated guinea pigs always had bacteria in the blood whether the inoculation material contained anthrax bacteria or not.

Other French investigators denied that the anthrax bacillus was present in the blood other than as *a by-product of the disease.*

Bollinger in summing up the facts known in 1872 was evidently convinced that the small rods which occurred in the blood were bacteria, were plant-like in their nature, and were the cause of the disease.

Davaine showed that the virus was destroyed by a temperature of 48 to 55°C., and that he could kill it in very dilute poisons such as chromic acid in a solution of 1 part in 1,000, 2,000, and up to 7,000, easily with all the dilutions up to 6,000, after which the action became doubtful. Experiments with iodine gave similar results. The fact that the stronger dilutions of the antiseptic killed, while the weaker ones enabled the organism to escape unharmed, convinced him still further that he was dealing with a living body which could be poisoned, and not with a chemical, which should have shown the same reaction with any degree of dilution. The reaction to heat further strengthened this conviction and he quoted from the article "Bacteria" in the dictionary on the medical sciences where Pasteur's investigations on the organisms which rot vegetables had been shown to be all killed at a temperature of 52°C., while Claude Bernard had shown that the blood of mammals is destroyed by a heat of only 45°C. As a temperature of 48 to 55° was necessary to kill this virus, it was not of the blood itself but was a bacterium.

Davaine[13] now interested another noted French scientist, Pasteur, in this disease. Pasteur had studied the organisms which produce the souring of beer or wine, and had investigated a bacterial infection of the silk worms. Davaine records that, in order to refute the statement of some of these opponents that the organisms were not always present in the blood of animals dead from the disease, he had a rabbit, dead of the disease, examined by a committee composed of Pasteur, Claude Bernard, and his two opponents. The organisms were found in the greatest abundance in the body of the animals, as Davaine had contended they always would be.

The refutation of the charge that the organisms were only present in the animal after the disease had made some headway could be easily disposed of by the inoculation of animals that were entirely healthy and which gave no indication in the blood of any organisms of any sort. The contention that the products of the disease were propagated by some invisible substance or substances in the blood which propagated it, if some of the blood were introduced, was a more difficult matter and could be answered only by using some substance which did not come from the animal. As long as even an infinitesimal amount of the blood was inoculated, the charge might still be made that the blood was the real cause and the organisms only incidental. Koch later answered this objection very neatly by growing the organism outside the body of the animal, transferring it repeatedly so that none of the original blood was carried over; then making successful inoculations from the eighth transfer. Davaine[20] had made dilutions of blood up to a millionth of a drop and secured the disease, but Koch's method was very much more conclusive as a proof.

Davaine published continuously on the disease between 1858 and 1874, showing that the disease could be carried by the blood and was invariably present in the blood

of infected animals. Two new names then began to appear in the discussion, Pasteur and Koch, and they led the discussion and investigation during the latter part of the 'seventies, but it must be remembered that all the groundwork had been laid before they appeared. Davaine missed the essential point; he always used blood or diluted blood and did not try to cultivate the organism entirely outside the body in materials which were not blood.[10–23]

Cohn and Koch.—Ferdinand Cohn,[8] professor of botany in the University of Breslau, Germany, had investigated in detail spore formation in the hay bacillus and arrived at the conclusion that if a liquid, such as a hay infusion, were boiled and organisms developed in it, they were due to species of bacteria which formed spores, especially *B. subtilis*. He knew that spores were very resistant to boiling water not only from his own experiments but from those of Roberts,[36] and that spores were the

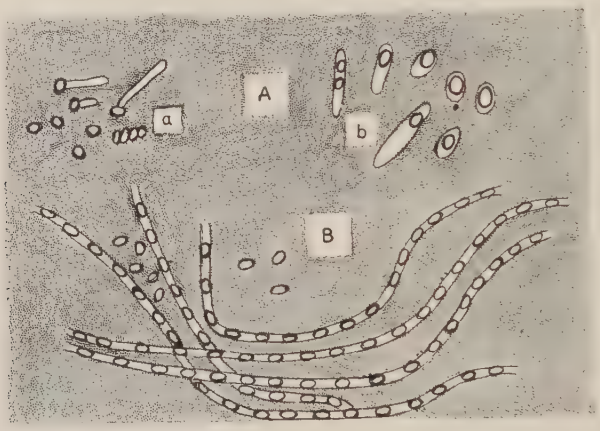

FIG. 124.—Anthrax organisms from original drawings by Koch and Cohn: *A*, spores; (*a*) germination; drawn by Koch, (*b*) drawn by Cohn; the two magnifications seem to have been different; *B*, spore formation in 24-hour culture, some cells in the filaments have broken and released the spores.

only parts of bacteria that could stand such treatment. He had already suggested that the organism in the blood in anthrax would be found to be also a spore producer. So he was pleased, he states, when he received a letter in 1876 from a Dr. Robert Koch, unknown physician, practicing in the little town of Wollstein, about 75 miles from Breslau, that he had discovered the spores of the anthrax bacillus and had been able to grow it in cultures. Koch came down to Breslau and spent 4 days demonstrating his methods and results to Cohn and his associates, convincing them of the correctness of his conclusions. Some of the drawings which Koch published were made by Cohn's own hand (Fig. 124), and the publication was made in the personal journal in which Cohn's own investigations were given to the world.[9]

Cohn was at this time the best-known German investigator of the bacteria, and his sponsoring of the unknown doctor with his new ideas gave him a hearing and prestige that Koch otherwise might have waited years to attain.

Koch used the aqueous humor from the eye of an ox as a substance upon which to grow the anthrax bacteria and was surprised to find that the filament increased in length so rapidly that in a few hours it covered the microscope slide. Inside these filaments were little ovoid bodies which were, as Tyndall expressed it, "like peas in a pod." He ascertained further that the organisms divide after they have attained a

certain length, that the spores are formed only in contact with the air, and that they germinate in aqueous humor if kept in an incubator at 35°C. for 3 to 4 hours. *And these artificial cultures, after inoculation, produce anthrax.*

Pasteur.—Pasteur started at once to work with enthusiasm on the bacillus, as the disease was of great importance to the sheep-raising industry of France and in many places had also become a menace to human beings, but he did not publish anything until the following year when he[34] issued a note on the growth and disease production of the organism in various animals. His first contribution was the proof that these anthrax rods would grow in culture media, other than that of animal origin, and would still produce disease. The liquid in which he grew it was his "yeast water" made from boiled yeast cells and inorganic chemicals.

DAVAINE, COHN, KOCH, AND PASTEUR

To Koch and Pasteur are given the credit of establishing the germ theory although Davaine had carried on similar inoculations with blood, and secured similar results. These two men, Pasteur and Koch, had, however, grown the organism *outside* the body in a culture fluid and from the generations which had appeared in the culture fluid had produced the disease. The only criticism that could be made against Davaine's demonstration would be (1) that he used the original organisms from the blood and (2) that he inoculated infinitesimal quantities of the blood.

The fact, too, must be remembered that Cohn[8] had already recognized that the organism producing the disease was a bacterium similar to those which he had been studying and growing in culture media, although not in pure cultures. He considered it to be very similar to the hay bacillus but non-motile. It would seem fair, therefore, to give credit to all of these men; to Davaine for recognizing the organisms and producing the disease by inoculation; to Cohn who recognized the organisms as bacteria of the same method of reproduction as ordinary species; to Koch who first grew the organisms outside the body of the animal in serum and caused the disease by inoculation; and to Pasteur who inoculated from similar cultures grown in broth or even yeast-culture solution and produced the disease. The whole matter of the production of disease by bacteria was in the air, and each of these men took one of the necessary steps to demonstrate the relation. All of this work appeared between 1870 and 1879 so that these years were very exciting ones; new discoveries piled up on each other and the students of the bacteria worked with feverish energy.

Pasteur's previous work on the fermentations induced in wine and beer by organisms introduced accidentally from the outside had prepared his mind and the minds of scientific thinkers for the acceptance of the germ theory. The fermentation induced in cider by the introduction of yeasts from the outside is not materially different from the infection of wounds or of the blood by bacteria. The cider develops alcohol from the activities of the yeast; the blood develops poisons which destroy the activities of the cells.

Pasteur definitely committed himself to the germ theory of disease in his famous lecture of Apr. 30, 1878, which he began with the proud exordium, "All sciences gain by mutual support." It must be recalled that he was addressing an assembly composed of scientific men of all sorts, and that among them there was a large proportion of medical men of the old school; that Pasteur had been elected as a mineralogist, and that his work had been along almost every scientific line except that of medicine, so that the opening words may be considered as an apology for venturing into the medical field, or, possibly, it was a challenge.

Pasteur set himself and his assistants, Chamberland and Roux, the task of solving some of the problems in connection with anthrax. Bert[2] had announced, for example, that he could kill all the anthrax organisms with compressed oxygen, and that the animal would contract the disease just the same whether the organisms were living

or dead, if the blood were inoculated. But when Pasteur grew the organisms in culture media, unlike blood, he disposed of the objection that a substance of animal origin was necessary to start the disease.

It was known, for example, that all the warm-blooded animals were susceptible to the attacks of the anthrax organism if it entered the blood, but that ordinary fowls were exempt. The reason for this immunity was not apparent. Pasteur denied that any animal, fowl or mammal, would allow the growth of the anthrax organism in its blood and challenged his opponent, Colin of Alfort, to produce a fowl dead of the disease. Colin took up the challenge but, in spite of a number of trial inoculations, was unable to produce anthrax. Pasteur twitted him with his lack of success and then generously offered to produce a fowl dead of the disease at Alfort, the home of his critic. The story is told in Vallery-Radot's "Life of Pasteur":

Passers-by were somewhat surprised to see Pasteur emerging from the Ecole Normale, carrying a cage, within which were three hens, one of which was dead. Thus laden, he took a fiacre, and drove to the Académie de Médicine, where, on arriving, he deposited this unexpected object on the desk. He explained that the dead hen had been inoculated with charbon two days before, at twelve o'clock on the Sunday with five drops of yeast water and that the hen had died twenty-nine hours after the inoculation. He also explained in his own name, and in the names of Messrs. Joubert and Chamberland, how in the presence of the curious fact that hens were refractory to charbon, it had occurred to them to see whether that singular and hitherto mysterious preservation did not have its cause in the temperature of the hen's body, "higher by several degrees than the temperature of the body of all the animal species which can be decimated by charbon."

This preconceived idea was followed by an ingenious experiment. In order to lower the temperature of an inoculated hen's body, it was kept for some time in a bath, the water covering one-third of its body. When treated in that way, said Pasteur, the hen dies the next day. All its blood, spleen, lungs, and liver are filled with bacilli anthracis susceptible of ulterior cultures either in inert liquids or in the bodies of animals. We have not met with a single exception.

As a proof of the success of the experiment, the white hen lay on the floor of the cage. As people might be forthcoming, even in the Academy, who would accuse the long bath of having caused death, one of the two living hens, a gray one, had been placed in the same bath, at the same temperature, and during the same time. The third one, a black hen, also in perfect health, had been inoculated at the same time as the white hen, with the same liquid, but with ten drops instead of five, to make the comparative result more convincing; it had not been subjected to the bath treatment. "You can see how healthy it is," said Pasteur, "it is therefore impossible to doubt that the white hen died of charbon; besides, the fact is proved by the bacteridia which fill its body."

A fourth experiment remained to be performed on a fourth hen. Could a hen, inoculated of charbon and placed in a bath, recover and be cured by merely taking out of its bath? A hen was taken, inoculated, and held down a prisoner in the bath, its feet fastened to the bottom of the tub, until it was obvious that the disease was in full progress. The hen was taken out of the water, dried, and wrapped up in cotton wool and placed at a temperature of 35°C. The bacteridia were reabsorbed by the blood, and the hen recovered completely. The mere fall of temperature from 42°C. to 38°C. caused a receptive condition.

All sorts of objections were raised; many of them with apparently much truth in them, but really founded on error. Pasteur's opponents in the Academy of Medicine claimed that the organisms which were found in the blood of the animals, dead of the disease known as anthrax, were there present to use up the products of the disease itself and were not the cause; others, that the organisms, if inoculated into the blood of any animal, would grow there and produce the disease.

He was kept busy answering objections and contriving experiments to convince the French scientific world that the organism was the active cause of disease, a very important work, as Paris, at that time, was the center of learning and the French Academy had a leading place in the scholarly world of Western Europe.

Pasteur and Antiseptic Surgery.—Pasteur was interested at this time, not only in anthrax and its organism, but also in *septicemia*, a condition which occurs in infected wounds in the absence of the oxygen of the air. *A septic infection* would mean the development and spread of organisms in the blood, that is, *septicemia*. The term which would now be more likely to be used for such a condition might be *bacteremia*, an infection with organisms but of a temporary nature. Pasteur recommended that surgeons should make use only of instruments, bandages, and sponges which had previously been heated to 130 to 150°C., and that the hands should be washed with the greatest care. In other words, use all the means which we associate today with the word, "*asepsis*," meaning "without disease."

Another term which came to be used was "*antisepsis*," (introduced by Semmelweis) meaning "against disease." This latter term is now applied to chemical agents which prevent the growth of bacteria and, hence, the "sepsis" or diseased conditions which they produce. The use of chemicals had already been developed in 1867 by Lister, as already noted, but this was a still further advance in surgical procedure at a time when the surgeon might go directly from a post-mortem to a surgical operation with only a hasty cleansing of his hands and his instruments, and when hospitals were regarded with such horror by the people that they were considered only the antechamber to the morgue, sometimes a third of the patients dying before they could escape. In some operations the mortality was 90 and even 100 per cent.[25] The maternity hospitals were especially dangerous, and the women of the lower classes of the city avoided them as they would the plague, although many were forced to make use of them. Pasteur offered an explanation for the high death rate and suggested the principles which would have to be followed if it was to be reduced. In many of his ideas he had been superseded by Lister in Scotland and England, but he introduced improvements in many ways, especially in the *prevention of infection*, while Lister attempted largely to control infection by the action of disinfectants.

VACCINATION AGAINST ANTHRAX

Pasteur continued to study the organisms of anthrax and others similar, with the intention of obtaining a culture which would produce the disease but in a milder form, in which he was so successful that he announced his discovery in 1881. He did not discover the method at once but arrived at it by the study of another disease, chicken cholera, where he could weaken the cultures by exposing them to the air for some time.

With the anthrax organism, it was necessary to expose it not only to the air but to abnormal temperatures in order to weaken it sufficiently not to cause death. At 45°C. it would not grow, but at 42 to 43°C. it grew but produced no spores and was much weakened. After 12 days at this high temperature the organism was so *attenuated* that it would not kill sheep when inoculated, but in order to be absolutely positive, the cultures were held for 24 days at that temperature before they were used for inoculation purposes. When returned to a low temperature, the virulence also gradually returned. The organisms at this high temperature produced no spores. Chaveau[7] found that, with the anthrax organism, the admission of oxygen made no difference in the attenuation (as Pasteur had once supposed), so he used high temperature alone. He also tried very high temperatures for a short time, 2 to 4 hours, during which the organisms were weakened and did not grow, although they were not killed.

The method finally adopted for use in the Pasteur anthrax serum was to grow the culture at 42.5°C. for 24 days and then use it for inoculation purposes on cattle or sheep. A second culture which had been subjected to this high temperature for only 12 days was then inoculated a few days later. With cultures used promptly, success with this method is assured, as can be seen by the famous test conducted by Pasteur, and told with dramatic force by Vallery-Radot:[39]

The numerous crowd moving towards the yard of the Pouilly le Fort farm looked like a mobilization of general councilors, farmers, physicians, apothecaries, and especially veterinary surgeons. Most of them were full of skepticism and were exchanging jokes and looking forward to the complete satisfaction of Pasteur's adversaries.

Pasteur, assisted by Chamberland and Roux, as well as a third student, Thullier, prepared the animals for the vaccination. The vaccination candidates and unvaccinated test sheep were divided under a large shed. Each of the 25 sheep selected received on the inner surface of the right thigh, five drops of the anthrax culture which Pasteur called the first vaccine. Pasteur then gave a lecture on anthrax in the great hall of the Pouilly farm, in clear, simple language meeting every objection. Showing no astonishment at ignorance or prejudice, he methodically described the road already travelled and pointed to the goal he would reach. Six cows were also vaccinated.

On May 17, a second vaccination was made with a liquid which, although attenuated, was still more virulent than the first. If it had been used at first, a half of the animals would have died.

On May 31, the inoculation with anthrax took place on 50 sheep and 10 cows. On June 5 at the latest, the results would be known, and if successful, there should be 25 survivors out of the 50 sheep inoculated and 6 cows, out of the 10 inoculated. Every member of Pasteur's family shared his anxiety, preoccupation, and hopes.

On May 31, everyone was at the farm. The veterinary surgeon shook the virulent cultures of anthrax vigorously in order to insure that they should be well mixed, as he had been warned that the deadly organisms were all at the bottom of the liquid and that the inoculations might be made from the top growth only. A triple dose was given and the virulent liquid was alternated between the vaccinated and unvaccinated animals. At half past three everything was done and a rendezvous was arranged for June 2 at the same place. The proportion of believers and unbelievers was changing; Pasteur seemed so sure of his ground that many were saying "He can surely not be mistaken."

On June 1, Chamberland and Roux went back to the farm to judge of the condition of the patients. Among the unvaccinated sheep, several were standing apart with drooping heads, refusing food. A few of the vaccinated sheep showed an increase in temperature; one of them had 40°C. but all preserved their appetites and all the unvaccinated sheep were getting worse and worse.

On June 2, when Pasteur arrived at two o'clock in the afternoon at the farmyard accompanied by his young collaborators, a murmur of applause arose which soon became loud acclamation, bursting from all lips. The carcasses of 22 unvaccinated sheep were lying side by side; two others were breathing their last; the last survivors all showed the symptoms of anthrax. All the vaccinated sheep were in perfect health. No success had ever been greater than Pasteur's. The veterinary surgeons until then the most incredulous, now convinced, desired to become the apostles of his doctrine. Two of them held, at once, an autopsy on the bodies of two of the sheep and the bacteria were to be seen in abundance in the blood. The 6 vaccinated cows were uninjured.

Pasteur was accompanied back to the station by an enthusiastic crowd, saluting him with a luxury of epithets contrasting with former ironies as the immortal author of the magnificent discovery of anthrax vaccination, and it was decided that the farm of Pouilly le Fort should bear the name of *clos Pasteur*.

Many disadvantages were found with Pasteur's method when in the hands of the ordinary veterinarian, however, some of which were that two vaccinations must be given; immunity did not show until 1 week or 10 days after the vaccination; the cultures were not good unless used exactly as prescribed; there were usually some losses from vaccination; and finally, the resistance of the animals to the disease was lowered during the period that the vaccine was acting. In spite of these disadvantages, between the years 1882 and 1907, 8,000,000 sheep and 1,300,000 cattle have been inoculated in France and many more in other parts of the world. The methods now followed by veterinarians avoid many of these dangers and inconveniences, their basis being the use of the blood of a horse immunized to the disease in the form of serum injected at the same time as a very virulent anthrax culture in the form of spores of the anthrax bacillus; 10 cubic centimeters of the serum is injected on one side of the animal simultaneously with 1 cubic centimeter of spore culture on the other side. Washburn[40] claims that the immunity obtained is as good, and lasts as long, as that resulting from the Pasteur, or any other method, and avoids their dangers. The serum introduced on one side neutralizes the poisons produced by the active culture of bacteria on the other side until the body cells of the animal are able to take care of them. Preisz[33] isolated from weakened cultures very weak strains and highly virulent ones. In inoculating such an attenuated culture, therefore, both sorts of organisms may be introduced simultaneously. Weakening the pathogenicity of the culture does not prevent the formation of spores; spore formation and virulence have nothing whatever to do with each other.

Source of Infection and the Survival of the Organism.—The infection with the organism occurs through the intestine from the plants growing on infected land. This fact was known to the veterinarians many years before the bacteriology of the disease was fully established, and we learn from Bollinger[3] that, in 1872, the body of an animal dead of the anthrax in upper Bavaria was, according to police regulations, to be burned, as less time-consuming than the deep burial necessary in the shallow soil of those regions. Feser[24] states that, in 1669, it was made obligatory to bury the body of animals dead of anthrax in a deep grave remote from the grazing lands. Bollinger was well acquainted with the fact that the bacteria in the animal were

destroyed if the body decayed, since putrefying bacteria have a very antagonistic influence on the anthrax bacilli.

The spores of the organism are highly resistant, so, if the vegetative rods pass into this condition, as they will if allowed access to oxygen, they serve as a constant source of danger. Such a condition may occur if an animal, dead from the disease, is opened and the blood allowed to dry on the soil or on the floor of the stable. The recommendation is always made today to burn the carcass unopened, or to bury it deep in the soil in quick lime, and fence the burial place. Busson[6] found the spores of anthrax to be strongly viable after having been dried for 17 years on threads of silk and to produce the same virulent form of the disease in white mice when inoculated, so that the spot where an anthrax-dead animal is buried remains indefinitely a potential plague source.

Pasteur[35] suggested that the organisms would find their way to the surface of the ground even if the animal were buried to any ordinary depth, by earthworms bringing up soil in their alimentary canals and depositing it in the form of casts around the entrance to their holes. The spores of the organism could then be carried as dust or to the stalks or leaves of the plants by the splashing of the rain.

Morris and Riley[32] inoculated sterilized soil with a virulent culture of the spores of the anthrax bacillus from agar slants and then grew corn, oats, rice, beans, Bermuda grass (*Cynodon dactylon* (L.) Per.), and Bull grass (*Paspalum dilatatum* Poir.) on it in pots. Portions of the plants were removed after they were 3 to 5 inches high, placed in liquefied agar, and the agar poured into a plate. The organism appeared on a large proportion of the sections taken from the lower part of the plants but only on their outside. Pathogenicity of the colonies was demonstrated by inoculation into guinea pigs.

Animals grazing over fields where anthrax animals had been buried would take quantities of this infected grass into the intestine, and, in the course of time, some of the germinated spores might find their way through the intestinal wall into the blood stream, and the animal come down with a general infection. The walls of the intestine are not impervious to bacteria; organisms can pass through them and leave no trace. It is rather hard to visualize the process, but probably the organisms are enclosed by pseudopodia-like projections from the inner wall of the intestine, which include them inside the intestinal cells. From this layer they could be passed along to the blood streams.

Cadavers buried in light soil to no great depth are very likely to be washed out by the rain, and the spores spread over the surface of the soil. Koch[26] had noticed the same peculiar fact that had been observed by veterinarians for years previously, the disease is much more likely to appear on heavy soil subject to overflow by streams at certain seasons of the year. On light soils, the organism seems soon to die out or to lose its virulence. Since such heavy soils usually produce good crops of hay or clover, some of the early theorists suggested that the death of the animals was the result of too heavy feeding on rich foods.

The Conditions for the Formation of Spores.—The two theories have been suggested to explain the formation of the spores in bacteria. Koch claimed that, for the successful formation of the spores, the following conditions were necessary: (1) a favorable culture medium, (2) a definite high temperature, (3) free access to the air. A superabundance of food led to spore formation.

Buchner[5] was certain that a *lack of food* was at the bottom of the stimulus to the spore formation. If a culture medium was continually renewed, no spore formation would result, but after a substratum had become exhausted, even though the organisms had an abundance of air and the proper temperature, spore formation would set in and could not be stopped. The addition of 2 per cent ordinary salt to the culture medium hastens spore formation so that it appears in 18 to 20 hours, as confirmed by Schreiber.[37] Spore formation occurs in bacteria when they are suddenly checked

at the height of their vegetative development; any factor preventing growth may induce it. A favorable culture medium stimulates an abundant growth, but spore formation begins only when food is running short after the organisms are at their maximum development. For the anthrax bacillus, oxygen is absolutely necessary and if not supplied it will not form spores.[38]

Koch vs. Pasteur.—Koch attacked the Pasteur method of vaccination, the rôle played by the earthworms in carrying the disease to the surface of the ground, the inoculation of fowls, and the purity of the cultures with which the Paris laboratory was working[39] (pp. 357 to 360). The two opponents met on Sept. 5, 1882, at an International Congress of Hygiene at Geneva; it will be remembered that the war between Germany and France had just closed, and animosities were still strong.

Pasteur summed up the work that had been done in his laboratory by himself and collaborators and defended the French methods. Koch declined to reply in full at that time. Later in the year, however, he answered Pasteur in a scientific article[27] which was one of the most important he ever wrote, for in it he presented his views as to the real conditions under which he considered an organism to be pathogenic and the rules by which this pathogenicity could be determined. These have been incorporated into the canons of bacteriology as Koch's postulates:

"My viewpoint may be briefly characterized as follows: It has not yet been shown that all infectious diseases are conditioned by parasitic microorganisms, so, in every instance, proof of the parasitic character of the disease must be offered. The first step in this proof is a careful investigation of the parts of the body changed by the disease in order to establish the presence of the parasites, their distribution in the diseased organs, and their relation to the tissues of the body. It is understood that for this investigation, every means which the new microtechnic offers is to be brought into service. The tissues and their humors, blood, lymph, etc., in a fresh condition, as well as without and with reagents, are to be investigated microscopically. They are to be dried on the coverglass and stained by various methods. The imbedded objects are to be sectioned on the microtome, stained, and the slides subjected to careful scrutiny with the use of the best adapted lighting methods and lenses. After this fundamental orienting, it is possible to decide whether microorganisms occur in the diseased parts and as to the parts where they appear in pure culture; whether, for example, they are to be found in the lungs, spleen, blood of the heart, etc. After their presence has been established and their pathogenic nature, that they are the cause of the disease which has arisen, can then be decided. For this purpose, they are to be grown in pure cultures and freed from all adhering portions of the diseased body. They are then to be inoculated back, if possible into the same species of animal in which the disease was observed, or into such animals as it might be expected to occur with recognizable symptoms. In order to make this clear, I will cite tuberculosis. First, through a microscopic investigation, I established that the diseased organs were filled with bacilli of a special staining reaction; these bacilli were then isolated into pure cultures out of places where they were not mixed with other species, and were purified in this procedure. Finally, it was demonstrated that by the inoculating back of such pure cultures into numerous animals of various species whose susceptibility to the disease was known, tuberculosis could be produced anew. A second very instructive example is offered by erysipelas in man. It has been known for a long time that in this disease the lymph glands of the skin always contain certain micrococci. But it was not yet proved that they were the cause of the disease. After Fehleisen had succeeded, however, in growing these micrococci in pure culture, excluding all chance organisms which

occur on the surface of the skin, and by inoculation had succeeded in producing in man typical erysipelas, there could be no doubt that these micrococci were the cause of the erysipelas; and that the latter is to be regarded as a parasitic disease."

These postulates have been summarized as follows:

1. The organism must be found in abundance in the diseased tissues.
2. It must be isolated into a pure culture.
3. On inoculation, it must produce the same disease and the same organisms must be found present.

How Many Organisms Are Needed to Infect?—The question of infection with a single organism or a number of organisms comes up in connection with any study of prevention. Is a single organism all that is needed to give the disease, or, is repeated infection with a mass of them essential for the production of the disease? Can the blood of a cow or sheep dispose of a single organism or a few organisms, but if a mass attack is made by a number of bacteria, will they get the upper hand and the animal become infected with organisms to such an extent that it dies or is seriously ill? Cheyne claimed that a single anthrax organism is sufficient to cause the death of a mouse, although certain other diseases, such as fowl cholera, to which the mouse is very subject, will require an inoculation of 10,000 to 30,000 organisms. This conclusion was partially confirmed by Barber[1] who found a single organism capable of producing the death of a mouse, although it only did so if taken directly from an infected animal and in a small percentage of cases. If taken from cultures, a much heavier dose was necessary. The following table will show the effect of an increase in the dose and of the use of organisms directly from another mouse or from cultures:

Number of organisms	From infected mice			From cultures		
	Number inoculated	Number dying	Percentage dying	Number inoculated	Number dying	Percentage dying
1	41	3	7½			
1 to 2	88	17	19			
2 to 3	36	6	17			
3 to 4	18	5	27			
4 to 7	29	4	13			
6 to 8	63	15	23			
8 to 12	63	19	30			
12 to 16	11	3	27			
16 to 25	9	3	33			
25 to 34	7	2	28	3	0	0
34 to 50	13	5	38	2	0	0
50 to 66	6	4	67	6	0	0
67 to 100				7	0	0
100 to 134				15	2	13
134 to 167				9	0	
167 to 334				16	2	12
334				4	1	25

The organisms for the above experiments were picked up and counted in capillary pipettes by the help of the mechanical device which Barber had perfected. The material obtained from living animals was much more deadly than that from cultures, but even then not all animals inoculated from animals died of the disease, as the mouse's body disposes of small numbers of virulent and of greater numbers of weaker ones. When the inoculated numbers reached more than two or three, however, the percentage of the deaths rose, but never did *every* infected individual die. The *individual immunity* of the mice was strong enough so that about three-fourths of them could destroy the organisms even when 20 to 50 of them were introduced directly into the blood stream. The cultivation on artificial culture media rendered the organisms much more easily killed in the body of the mice. These cultures were not old isolations but were simply transfers for the first time from the blood. A comparison in the same manner of the old cultures, kept for 30 or 40 years, with similar ones of a few months would be interesting. It is an old experience of all laboratory workers that bacteria usually lose their virulence in cultivation on agar or in broth.[29] The food conditions enervate them so that they are no longer virulent. Such cultures are said to be *attenuated*. Pasteur made use of attenuation to secure a strain that could be used for purposes of inoculation, hastening it by growth at abnormally high temperatures. The virulence of the organisms can be increased by passing them through a fowl that has been chilled a number of times. The fowl is very resistant to the organisms and probably only the more vigorous and virulent individuals survive.[28]

IMMUNITY

The pathogenicity of the anthrax organism illustrates the fundamental principles of *immunity*.

Some animals are very *immune* to the disease and will not contract it unless under unusual conditions of stress when the body resistance is lowered, as in the case of the fowl, kept wet and cold for several days.

Certain individuals are difficult to infect, as in the case of the mice; they have an *individual resistance*. In a similar manner, some races are more resistant than others.

The cause of these differences in *susceptibility* and *resistance* is not known; we do know some of the conditions which will produce one or the other, but as to the internal body changes, such as in the blood, no adequate explanation has been offered.

How Does the Body Free Itself from Invaders?—The problem of the disposal of bacteria by the body of an animal has interested bacteriologists and biologists since it was shown that they could produce disease. The most interesting of the theories proposed has been that of Metchnikoff, a Russian zoologist (Fig. 125), who worked for years on the absorption of food particles, especially smaller plants and animals, by the protozoa and crustacea. The theory which he evolved in this study of an apparently useless subject was carried over into bacteriology,[31] and is one of the most interesting, suggestive, and helpful hypotheses propounded to explain the method by which the body frees itself from intruding foreign organisms.

If the body of a sponge, of a sea anemone, a worm, or a crustacean is invaded by foreign organisms, the amœboid cells come to the point of invasion and engulf them.

These smaller animals are many of them so transparent that the whole process of engulfment and digestion can be followed in detail (Fig. 126). This property of absorption and intracellular digestion of the bacteria or other small forms is one of the ordinary feeding habit of the amœboid and infusorian protozoans. Some of the cells of the higher animals retain this ability to take in the smaller forms, and these cells, since they wander over the entire body in the blood or plasma, have been called, by Metchnikoff, *phagocytes* (wandering cells). These wandering cells not only eat the invaders of the body but absorb or replace any old cells that have lost their function. He divided the phagocytes into two types: (1) the large fixed mononuclear cells of certain organs of the body; (2) the motile amœboid cells, many of which are polymorphonuclear but others are mononuclear. He has called the large cells, which have to do with the destruction of tissues, the *macrophages;* the smaller ones, mostly the polymorphonuclear whose function is to destroy the bacteria which enter the body, the *microphages.* These phagocytes, according to the Metchnikoff theory, are the soldiers of the body, cells which have retained all the old capabilities and appetites of the amœba, which, when the call to arms arrives, hasten in countless swarms to the invaded region to absorb the bacterial enemies or to lay down their lives in defense of the cell community.

Fig. 125.—Élie Metchnikoff (1845–1916). Discoverer of the rôle of the white blood corpuscles (phagocytes) in destroying bacteria; Director of the Pasteur Institute at Paris, after the death of Pasteur. (*Courtesy, G. P. Putman's Son's, publishers Metchnikoff's works.*)

The opponents of the phagocyte hypothesis, on the other hand, point out the fact that these phagocytes pick up not the active and virulent individuals but only those which have been weakened by the poisons which the body cells have thrown out. Instead of being the soldiers of the body, they are only scavengers who trail along when the eating is good and try to pick up a meal from the bodies of the weakened bacterial invaders. This attack on the phagocyte theory is repelled by its advocates with the experminental evidence that anthrax organisms will still cause the death of an animal if some of the containing phagocytes are introduced into the blood stream of another animal.

Inside the body of the phagocyte, the bodies of the bacteria undergo gradual disintegration in the little vacuoles in which they come to lie, the secretion of digestive juices transforming these vacuoles into rudimentary stomachs. The bacteria, stained with the usual technic, indicate all stages in disintegration by the reaction of the organisms to the dyes; the organisms recently swallowed still stain intensely and uniformly; those whose bodies have been attacked by the proteoclastic enzymes color less brilliantly and appear granular. These endoenzymes which digest the bacteria are never secreted outside the body of the phagocyte itself. The phagocytes are in active operation at all times in all the organs of the body but particularly on the mucous membranes, where the bacteria are likely to find lodgment, food, and growth. We take into the body at all times great numbers of bacteria in the food which we eat and the air which we breathe. The bacteria which lodge in the lungs might grow and be a serious menace, if it were not for the constant attention which the phagocytes give to invaders. All sorts of lung infections might occur if it were not for their helpfulness. The problem is to keep them working full time, and able to cope with any invader. We know that the body is not constant in its ability to repel

the invaders, and if the phagocytes are the means by which the bacteria are killed, we should expect the blood to vary at different times.

Opsonins.—This fact led Wright[42,43] to the conclusion that the serum deposits some substance on bacteria which induces the phagocyte to eat them. These substances he named *opsonins* (opsonin, in Greek, I cater for; I prepare food for). They prepare the bacteria in some way for the ingestion by the phagocytes. According to Wright, therefore, the opsonins would be the determining factor in immunity. The Metchnikoff school insists that the opsonins are the products of the phagocytes

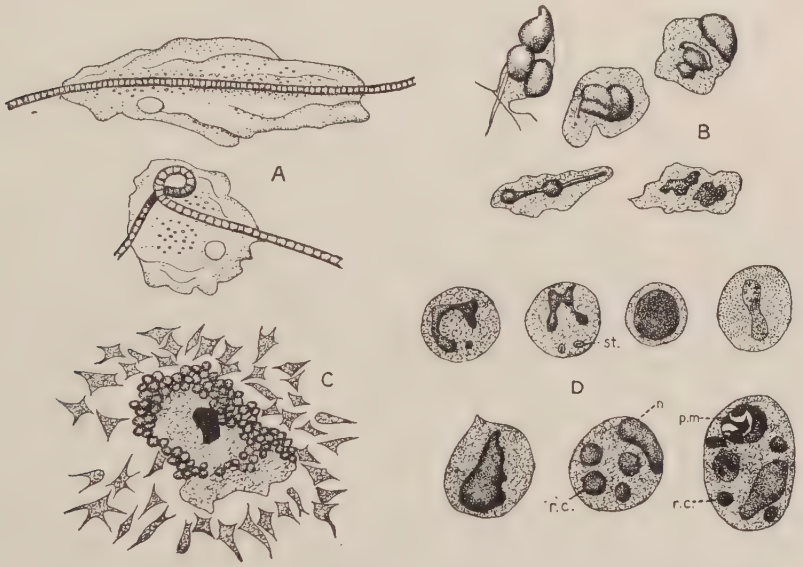

FIG. 126.—The absorbtion by amœba and leucocytes of various foreign bodies: *A*, an amœba incorporating a filament of a blue-green alga (Oscillatoria); *B*, spores of *Monospora* being injested and dissolved by the leucocytes of Daphnia; *C*, leucocytes surrounding an invading body in the larva of a sea-anemone; *D*, different types of leucocytes: *a*, polymorphic; *b*, polymorphic microphage with included streptococci, *st*; *c*, lymphocyte; *d*, cosinophile; *e*, mononeuclear macrophage; *f*, macrophage absorbing red blood corpuscles, *r.c.*; *g*, macrophage taking up polymorphic microphages, *p. m.* and red blood corpuscles, *r.c.* (*A, after Rhumbler; B and C, after Metchnikoff; D, after Burnett.*)

themselves and that they prepare the bacteria which they eat. Wright developed a method for determining the relative amount of bacteria which the phagocytes would eat as compared with a normal standard. One divided by the other gave the *opsonic index.*

The condition of the leucocytes in the blood of a person suffering from a disease may be determined by taking a little of the blood, washing it with a citrated salt solution until the leucocytes are free from all blood serum, allowing the washed leucocytes to remain undisturbed for 30 minutes. After this they will be at the top of the liquid and may be pipetted off and mixed with a carefully prepared suspension of the organism of the disease. The leucocytes and the bacteria are incubated for 15 minutes with frequent mixings when a little of the mixture is dried and stained as a film on a glass slide. The number of organisms taken up by 50 leucocytes is then determined by examination under the oil immersion lens and the average number per leucocyte computed. The same procedure is followed to determine the number of organisms per leucocyte in the blood of a person in normal condition. If normal

leucocytes take up 6.5 bacteria and the leucocytes of a patient suffering from a disease take up an average of 7.6 organisms, the opsonic index would be $7.6 \div 6.5 = 1.17$. The results seem to vary greatly in the hands of experienced technicians, and the phagocytic power of the leucocytes varies with respect to certain bacteria, even in the same individual. While the method has considerable diagnostic value in some diseases and has been used by Wright and his associates as a means of determining the condition of the blood, a very high opsonic index being advantageous in order to prevent incurring disease, it has not come into general hospital and clinical use.

Pleomorphism in the Bacteria.—The older authors made no systematic attempt to classify bacteria as far as species until after Cohn (1870 to 1890), when not only genera but species were recognized as distinct and permanent. Not all investigators believed in the permanence of bacterial species; some claimed that only one species of organism existed and that the modification of this organism produced all rod, coccus, and spirillum forms, this organism being *pleomorphic* (pleo = many, morphic = formed). The permanence of bacterial species was of vital importance to the germ theory of disease and any attempt to show that pathogenic bacteria were only strains of ordinary saprophytic forms was violently attacked by the new school of pathogenicists.

B. subtilis and *B. anthracis* resemble each other in many morphological and cultural characters (as Cohn[8] had noted) although the former is motile, the latter non-motile. The shape of the cells, the spore formation, and the spore germination are very similar. Buchner[4] was convinced that he could convert the anthrax organism into the hay bacillus by growing it for a long period of time in liquid culture solutions. The organism was transferred regularly, and after the one hundredth transfer and the seven hundredth generation, he began to notice a tendency of the bacteria to grow on the surface of the liquid, a condition which became still more pronounced after the nine hundredth generation and 90 days. After the thirty-sixth transfer, the organism lost its infectiousness and was considered to be the ordinary hay bacillus. The very strong probability is, of course, that, after these numerous transfers, he did have the hay bacillus, but that he had accidentally introduced it.

To make the demonstration complete, it would be necessary, too, to convert the hay bacillus into the anthrax type, but this was found to be very difficult. Inoculations with hay organisms into mice were without positive results other than to cause a general septicemia if a very large quantity were used. He claimed to have been able to transform it, but no one has succeeded in confirming his results.

The work of Buchner was immediately attacked by Koch who accused Buchner of being simply a follower of Nägeli, the principal exponent of variability of bacterial species, and of having obtained results that were in accordance with theory and not with the facts. Nothing further was heard of transformations of this sort and science has accepted the Koch theory that pathogenic organisms are distinct species, not derived directly from, nor convertible into, ordinary saprophytic forms.

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CHAPTER XXVIII

BACILLUS TYPHOSUS

(THE TYPHOID FEVER ORGANISM)

(Eberthella typhi)

The organism of typhoid fever was discovered very soon after the anthrax bacillus had been shown to cause the disease known as anthrax or splenic fever. The marvelous activity manifested at this time in bacteriology is indicated by the fact that the organism was observed in the lymph nodes, in certain patches in the intestine, and in the blood vessels, at just about the same time by three investigators, all working separately, Eberth, Klebs, and Koch, all of whom published within two years, 1880 and 1881. It remained for Gaffy to isolate the organism in pure cultures in 1884, although even here he may have been preceded by Klebs who isolated on gelatin an organism which he had seen in numbers in the lymph nodes in the mesentery. A few years later, the same organism was isolated from the stool of affected patients, later, from the blood of patients and from the rose spots which appear on the abdomen during the disease. Since then the organism has also been obtained in pure cultures from water supplies known to have been contaminated.

The organism is a short rod, 1 to 4 microns × 0.5 to 1 micron; motile by 5 to 7 flagella around the outside, with no spores. In cultures it is very similar to the colon bacillus, being one of the members of the colon-typhoid group (Fig. 127).

B. typhosus is only parasitic if taken through the mouth into the digestive tract where it may cause inflammation and ulceration of certain portions of the alimentary canal known as *Peyer's patches*, tissue regions named from their discoverer, found on the small intestine. Their lymphoid structure makes them different in texture from other portions of the intestine.

The Disease.—Typhoid fever is primarily an intestinal disease but the organisms spread throughout the body in the course of the infection. The disease has been divided by Gay[4] into four more or less distinct phases. (1) The incubation period, lasting about 7 days, is characterized by headache, listlessness, pain in the back or legs, nausea with sometimes vomiting and a chill. (2) The increasing fever stage covers 1 week or 10 days, during which time the fever peculiar to this disease makes itself manifest. The temperature rises in the afternoon to 103 to 104°F., at times, but may fall during the night almost to normal. The headache may become worse and either severe constipation or diarrhea may be present. At this time, the disease shows an inflammation of the intestine, and the liver and spleen become enlarged. (3) The level fever stage during which the fever may go to 103 to 105°F. and the decline in the morning is not so marked as in the preceding stage. The headache becomes less severe but dullness and stupor take its place and delirium may develop

at night when the fever is high. Red patches, known as *rose spots* appear on the abdomen in the majority of cases. The patient loses rapidly in weight, owing to the fact that no solid food can be taken or digested, as ulcers of considerable size are formed on the intestinal wall on the Peyer's patches. The difference in tissue structure gives opportunity for the bacteria to grow in them, the lymphoid tissue being easily infected (Fig. 127). The glands, spleen, and liver, much swollen during the early part of this stage, gradually reduce in size to normal. Perforations of the intestinal wall by the ulcers or hemorrhage, if the ulcers should happen to be near a large blood vessel, are complications to be feared at this time and are the cause of many of the deaths from typhoid. (4) The period of diminishing temperatures should nor-

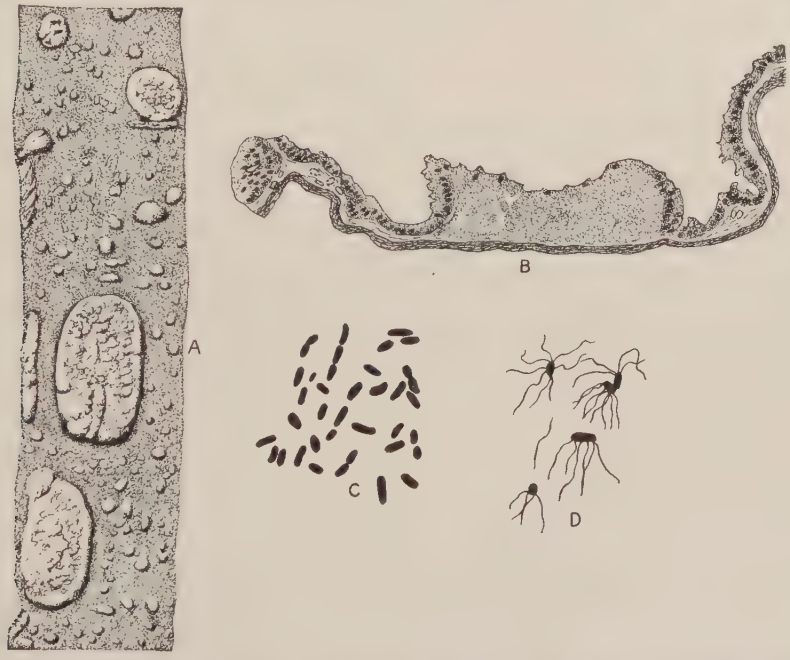

FIG. 127.—*Bacillus typhosus* and its lesions in the intestine: *A*, early stage in inflammation of ileum with Peyer's patches much swollen; *B*, solitary gland of intestine swollen and without its mucosa; *C*, the organisms; *D*, stained to show the flagella. (*A*, *after Nichols; B, after McFarland; C, after Almquist; D, after Migula.*)

mally occur from about the third week. The temperature no longer rises so high in in the afternoon. The ulcers on the intestine heal, until they entirely disappear but danger of perforations and hemorrhage are still present. As the return to normal temperature is no certain indication that the ulcers are healed, no solid food is given for some time after the temperature is normal and shows none of the oscillations characteristic of the early stages. The whole course of the disease, as outlined, occupies 5 to 6 weeks if no relapse occurs, due to the infection of other spots on the intestine with a recurrence of rythmical fever. Recovery from a relapse is not so long as from the original attack, although a relapse is really a recurrence of the disease. The mortality from relapses is very low.

The Incubation Period.—The incubation period of the organism (the time which elapses between the entrance of the organism into the body and the appearance of the disease) is rather difficult to determine and seems to vary from about 1 week in some

cases to 3 weeks in others. With most natural infections it is difficult to determine the exact time at which the bacteria were taken into the body, but records made by Kisskalt[8] of 50 cases, most of which were accidental and in the laboratory, would lead to the conclusion that the ordinary average incubation time was about 2 weeks and that the number of organisms which were swallowed made little difference in the length of time required for the production of the disease. An epidemic caused by eating contaminated spaghetti in which the organisms had increased in numbers before it was served, affected 93 people. Over half of the cases showed symptoms before the eighth day, the first one appearing on the third day, 12 on the fifth, and 19 on the sixth. The older idea was that the organism required 10 days of incubation, but Vaughn[14] believes that the time varies with the dosage, a large number of organisms shortens the incubation period since the larger part of it is utilized for bacterial increase in the intestine.

The Spread of the Organism in the Body.—While the organism occurs in numbers in the intestine and in the mesenteric glands, liver, spleen, etc., it occurs in the blood throughout the body while the disease is in progress and disappears from it as the patient recovers. The organisms seem to enter the blood from reserve foci in the body, presumably the glands, until destroyed and the entire body is freed from them. Their destruction in the blood is the result of the attack of antibodies which kill the organisms but leave their bodies to be disposed of by the blood. The proteins in the body of the organisms are of a different composition from those of the blood and, as they are absorbed, marked reactions occur in the patient,[15] since foreign proteins of any sort (such as white of egg) when injected cause similar disturbances. These disturbances are in the form of *fever*. As long, therefore, as the bacteria are being destroyed and absorbed, the body responds by an abnormal temperature, so that the temperature can be considered as a measure of the destruction of the organisms and with the return of the temperature to normal, the typhoid organisms have been largely destroyed and absorbed.

Largely a Human Disease.—Typhoid fever was for a long time believed to be a disease to which mankind had an exclusive monopoly as all attempts inoculating any of the lower animals, even apes and monkeys, had failed. Metchnikoff and Besredka[11] finally succeeded in inducing typical typhoid in 15 out of 16 of the higher apes which had been fed on the stools of typhoid-fever patients. Up to this time the supposed typhoid fever organism did not fulfill all of Koch's postulates, since it was impossible to get any subjects for inoculations. Its pathogenicity had been fairly well established, however, by accidental inoculations around laboratories and hospitals; from the typhoid organism often kept in stock cultures for tests on the blood of typhoid fever patients, laboratory workers became accidentally inoculated. Metchnikoff and Besredka were successful, not only in producing the disease in apes but also in showing that the filtrate from the stools of patients having the disease would *not* produce the disease, indicating that it was organisms and not a filterable virus such as occurs in some other diseases. These higher apes had the characteristic run of fever, diarrhea, and blood tests exactly the same as human beings, and communicated the disease to others of the same breed.

The Isolation of the Organism.—Even in pronounced cases of typhoid fever, the colon organism is the predominant type. The skill of laboratory workers has been put to the test to distinguish between two races so similar in morphology and in their reactions to many of the ordinary laboratory tests. The colonies are so similar on the ordinary media that it would be impossible to distinguish between them without the addition of chemicals or dyes. The media usually employed are the Conradi-Drigalski, Endo's, or eosin-methylene blue. These media contain a color affected by the acid produced by the colon bacillus but not by the typhoid organism which is not an acid producer.

Endo's medium is made from nutrient agar with the addition of lactose, basic fuchsin, and sodium sulphite. The agar as it is used in the plates is colorless since the sodium sulphite suppresses the color of the red fuchsin. After the colonies appear, the acid production of the colon organism changes the reaction of medium in its immediate vicinity, the colony becomes red and develops a metallic sheen due to the production of an aldehyde. The typhoid colonies, not being acid producers, are colorless. Suspected colonies obtained by these methods may then be subjected to further tests, such as the coagulation of litmus milk, the reduction of neutral red, or the fermentation of various sugars.

The most conclusive test, however, is the agglutination of the typhoid bacteria by the blood of a typhoid fever patient or of a person who has recently recovered from the disease. This test, known as a "Widal" from its perfecter (although the credit really belongs to Gruber) can be used for two purposes: (1) determining the identity of a suspected organism, if the blood of a patient who has the disease or serum from an immunized animal is available, or (2) ascertaining whether a patient has the disease. The technic of the test is very simple but must be carried out by an experienced laboratory bacteriologist. A drop of the blood of the patient is diluted with 30 to 80 drops of sterile distilled water and some typhoid bacteria from a culture are mixed with it and at once examined under the microscope. If the blood is that of a person having the disease, or who has recently had it, the bacteria begin in a few minutes to clump in groups of varying numbers (Fig. 128). In the blood of a person sick with typhoid or recently recovered from it, typhoid organisms adhere when they touch each other, *agglutinate*. No other organisms will do this in typhoid blood, nor will any other blood, except that of a typhoid case, cause agglutination. These facts make the test of the greatest value in determining the organism or the disease in occasional cases where the clinical diagnosis of the fever is not clear. It is of no value, however, on patients who have been immunized against typhoid by the injection of typhoid vaccine, as all react positively. The very prevalent use of such vaccination has made the Widal reaction much less important than formerly.

FIG. 128.—Agglutination in *B. typhosus*, (semidiagrammatic). In serum from individuals who are not immune, the distribution throughout the field is homogeneous; in immune serum, the bacteria gather in clumps.

Paratyphoid Fever.—Typhoid fever sometimes appears in mild forms, often difficult to distinguish from other fevers. The old, so-called "walking" typhoid, where the patient was troubled by a diarrhea and general weakened condition, but with less serious consequences, was frequently of this type. The mortality is estimated as low as 1 per cent, according to Gay, but Labbe, quoted by the same author, compiled statistics that show that the relative mortality in typhoid and paratyphoid is, according to one series of records, 25 and 5 per cent, and, according to a later one, the proportions are 15 and 11 per cent. These figures are from French sources before 1915 and are surprisingly high for typhoid as well as paratyphoid. The danger from relapses, complications with pneumonia, etc., is the same as with typhoid, but the mortality is usually less.

The organisms associated with paratyphoid are a group which has been divided into *B. paratyphosus Alpha* and *B. paratyphosus Beta*. The paratyphoid group

stands midway between coli and typhoid types. In cultural reactions, paratyphoid bacteria are more nearly like typhoid in that they react the same on Conradi-Drigalski agar and Endo's medium, form no acid or indol, but produce gas with sugars, and give the coli reaction in the neutral red, glucose-broth fermentation tubes. They are more difficult to isolate from the blood than the typhoid organism, their appearance there being of rather short duration. The agglutination test by which most bacteriologists identify typhoid organisms is not nearly so certain as with the true typhoid organism. One type of paratyphoid, the Beta, is more readily agglutinated than the Alpha. The serum from a patient having an infection with the Beta organism will give an agglutination with typhoid organisms also. About 8 to 10 per cent of true typhoid cases give a positive agglutination test with one or both paratyphoid strains.

Typhoid-fever Carriers.—The typhoid-fever organism is usually eliminated from the body with the return to a normal temperature but a small per cent of the recovered patients continue to harbor it for periods of 3 months to 1 year. These infected individuals while in entirely normal health themselves may be a source of danger to all with whom they associate, as the organism may be carried from the hands of such "carriers" into the food of others. Investigations of convalescents from typhoid fever, as carriers, have led to the conclusion that between 2 and 10 per cent of all who have had typhoid fever remain for some time infected with the organism; the majority of the reports indicating about an average of 3 to 5 per cent. With 150,000 cases of typhoid in this country and 5 per cent of them carriers, it would mean that 7,500 carriers are free every year to spread the disease.[4] The larger part of the carriers give off the organisms in the feces, probably from an infection of the gall bladder, but occasionally the urine may be infected from organisms which persist in the kidney or in the urinary bladder. The importance of the carrier as a source of the disease was first pointed out by Robert Koch[9] and persistant work by a number of bacteriologists in various countries have confirmed and enlarged his theories. Many carriers cease to spread the organisms after 3 months but a number continue to throw them off for indefinite periods, some for many years. In addition to carriers who have had the disease, however, a small number of persons who have never had the disease have an intestinal infection with *B. typhosus* and are a menace to the community all their lives. The organism lives a purely saprophytic and harmless existence in their intestines, but, introduced into the food of others, may induce typhoid fever. Dealing with such innocent but dangerous individuals is one of the most puzzling of modern sanitary problems. We may have in our midst individuals who have broken no law but who are as dangerous as the worst criminals. It is unjust to imprison them or to confine them to a hospital or sanatorium, although best for the community. The measures taken must be largely with the cooperation of the carriers themselves to obey certain regulations, such as the entire abstention from the handling of any food, especially lettuce, fruit, etc., which may be eaten raw; to disinfect the hands thoroughly; to disinfect the bed linen and undergarments in an antiseptic solution; and, if possible, to disinfect the excreta. The lot of the permanent typhoid carrier is hardly one to be envied.

The Spread of Typhoid Fever.—The spread of typhoid may be either as isolated cases in families or as an epidemic, but cases originating from carriers are usually of the individual or family type. If a milk or water supply becomes contaminated, a milkman's route may show many cases or a whole community may come down with the fever. The tracing of the source of infection in typhoid fever is one of the most fascinating phases of modern preventive medicine. If a number of cases of typhoid should occur in a small city, they would be tabulated to discover the oysters, milk, ice, or vegetables, which had been bought in common, since it is by means of such food materials taken into the body through the mouth that the disease is carried

to a number of individuals. If only a single family or individual contracts the disease, infection may have occurred while on a trip or from a carrier. Some carriers have been cooks and have carried the disease into families in the food which they prepared for the table. Such carriers should be controlled and their handling of food stopped, so that individual cases are studied as well as epidemics, as they may give the clue to unsuspected carriers.

The smaller epidemics are milk-borne, while those where hundreds are attacked are more likely to be the results of infection of the drinking water.

Water-borne Epidemics.—The Plymouth, Pennsylvania, epidemic of 1885 is the first large water-borne epidemic where the means by which the organisms found their way into the water supply is known. This little Pennsylvania mining town of 8,000 inhabitants had 1,104 cases and of this number 114 died. The town was supplied with water from a stream of mountain origin with very few inhabitants along its water shed (Fig. 129), the water being considered to be so pure that dams were thrown across the channel and the water pumped from reservoirs directly into the mains. The largest of these reservoirs, the one most distant from the town, contained 5,000,000 gallons; the second 3,000,000 gallons; the third, 1,700,000 gallons; and the small distributing reservoir near the town 300,000 gallons.

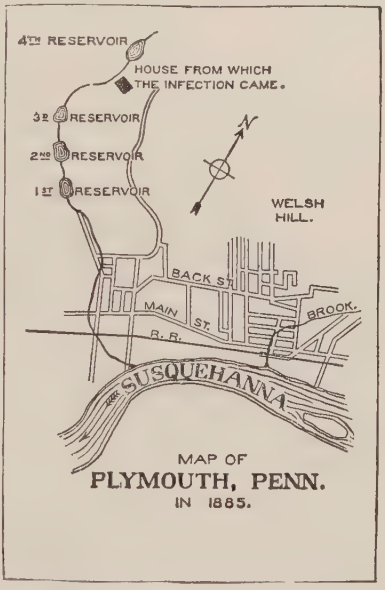

FIG. 129.—Sketch map of Plymouth, Pennsylvania, showing the position of the reservoirs and the house from which contamination came. (*From Frost and McCampbell's "General Bacteriology."*)

A case of typhoid fever had occurred on Jan. 2, 1885, in a house situated just below the upper reservoir. The patient was sick until the middle of April, the case a severe one with a relapse. During the illness, the night dejecta of the patient were thrown behind the house on the snow without being disinfected, as the physician believed that the house was so remote that there was no danger of spreading the disease and was unaware that the stream, which flowed only a few hundred feet away, supplied the reservoirs below. The day dejecta were emptied into a privy almost on the surface of the ground, which would empty into the stream as soon as a thaw came. During the latter part of March, the snow melted and carried the dejecta into the stream which fed the reservoirs. The town had been supplied from the two lower reservoirs but as they became empty, the water superintendent allowed water from the second of the reservoirs to flow into the pipes in the last week of March. The first case of typhoid fever occurred April 9 and after April 12, the disease spread so that 50 to 100 new cases were reported daily. All classes of people were attacked and in all parts of the town. The epidemic demonstrated that the typhoid organism could survive freezing for some days (possibly months) in ice or snow and still cause the fever. It further emphasized the importance of disinfecting the dejecta of a typhoid fever patient even in a case far from any apparent danger of infection.*

Military camps always have an unusual amount of typhoid in spite of modern sanitation and knowledge of the typhoid organisms. During the South African Boer War, the English troops had 8,000 deaths among the 230,000 men, and during

* For other water-borne typhoid epidemics see Whipple.[18]

the Spanish-American War, 20,738 cases of typhoid fever and 1,580 deaths occurred among the 107,973 men in American concentration camps. The long detention of a mass of men in one situation leads to a thorough contamination of the ground and gives an opportunity for the organism to spread, and this infection, combined with unusual and often poor food, loss of sleep, mental strain, etc., makes soldiers especially susceptible.

Not nearly all who are exposed contract the disease; as with most of the so-called "infectious diseases," the person must be in a susceptible condition. In the Plymouth epidemic, it will be noted that, although all the inhabitants of the town must have used the same water day by day, only about one in eight of them fell sick. To produce the disease, not only the organisms but also the proper substratum for them must be supplied.

Resistance to the Disease.—A certain number of persons are not attacked by typhoid fever, as already noted, since they have some form of resistance and are immune to the disease. Individuals who have had the disease are usually free from the disease for many months, although numerous exceptions occur. The nature of the protection which an attack in the disease may give is not clear, but the blood of a convalescent from the fever will give the Widal agglutination test, usually lost in 6 months to 1 year, although persisting in a small percentage for some years. The present opinion seems to be that this immunity is not due to the presence of antibodies floating in the blood but to the ability of the cells to produce antibodies when the organisms reappear in the body. If the blood were full of antibodies, it could be used as a serum to kill the organisms in the blood, but this has been found impractical after the patient has entirely recovered from the disease, although immunity can be conferred with serum while the disease is still acting.

Inoculation against the Disease.—The present method of typhoid inoculation is due to Wright[16] who used dead cultures of the typhoid organism. Wright was not the real originator of this idea, since the same method had been experimentally used by Chantemesse and Widal.[3] Wright and Semple, in 1897, first published the complete method.[17] Typhoid bacteria grown for 2 or 3 weeks in broth are killed by heating them at 63°C. for 1 hour and then preserved in 0.5 per cent of carbolic acid. The vaccine is tested for sterility and the toxicity determined. The number of bacteria are counted and the vaccine divided into doses of 750,000 to 1,000,000 of organisms in each. Other modifications of the method have been proposed but all are based on the same principle.

The present method is to give these dead bacteria in two to five inoculations under the skin, at intervals of 7 to 10 days. The number of bacteria recommended, with three inoculations, is 500,000, 1,000,000, and 1,000,000 bacteria, making a total of 2,500,000. It has not been found practical to give the organisms by way of the digestive tract. The experiments performed by Wright, where he fed 1 to 15 cubic centimeters of his vaccine, showed no bad effects except a diarrhea in vaccinated individuals, but the development of antibodies was not stimulated as with inoculation under the skin.

The effect of the inoculations is to produce *antibodies* antagonistic to the bacteria which will weaken, and probably kill them, the bacterial bodies being absorbed. The process is, theoretically, the same as that which occurs when a person is attacked by the virulent organism. The body cells are stimulated to produce antibodies which, in the course of the fever, gradually get the upper hand of the bacteria and destroy them.

Numerous attempts have been made to use a weakened culture of the living organism itself, but such cultures seem to be too dangerous. It would seem that, with the variation in the virulence of the different strains of the *B. typhosus*, it might be possible to isolate an avirulent type which could be used with safety. Castellani[2]

used such a strain, grew it in bouillon cultures, further weakening it by growing it for an hour at 50°C. before inoculation. The results after inoculation with it were excellent and he claims that the immunity, in some cases, lasted for 4 years while dead organisms give an immunity of only 1 to 2 years, some claim for 2½ years.

Typhoid vaccinations, although proposed by Wright in 1897, were not general for a number of years, the first extensive tests being made on the British troops who took part in the Boer War in 1900. Most of the available data are from army vaccinations and indicate that inoculation with dead bacteria, while not preventing the disease in all cases, will reduce the percentage of cases and of deaths. Vaccination is now practiced in all the great armies of the world and sufficient evidence of its usefulness has been gathered to lead medical authorities to make it compulsory in most of them at the present time, especially if the troops are to be sent into a new region. The typhoid organism presents a great number of strains, so that, for vaccination to be successful, the dead bodies from a number of isolations obtained from widely divergent sources or inoculation with the strains from the region to which the person is going must be used. Such a serum made from numerous isolations is said to be *polyvalent.*

The following table will show the effect of vaccination in the U. S. Army up to the beginning of the World War:

		Cases per 100,000	Number vaccinated	Army strength
1901	No vaccination	674		
1902	No vaccination	699		
1903	No vaccination	514		
1904	No vaccination	435		
1905	No vaccination	314		
1906	No vaccination	572		
1907	No vaccination	379		
1908	No vaccination	320		74,692
1909	Voluntary	335	830	
1910	Voluntary	243	16,093	81,434
1911	½ Compulsory	85	25,779	82,802
1912	Compulsory	31	all	88,478
1913	Compulsory	4.4	all	90,752
1914	Compulsory	7.5	all	92,877

In spite of universal vaccination, 885 cases occurred among the 1,900,000 American soldiers in France and of 270 proved cases all had received the vaccination, so that it cannot be considered as an absolute preventive.[10]

Typhoid Fever is a Seasonal Disease.—Typhoid begins to spread as soon as warm weather starts and keeps increasing in summer until its peak is attained in September, when the hot weather has ceased but the enervating effects are still to be felt (Fig. 130). The numbers then drop off to a minimum, usually in January and February. The low rate during the winter, as compared to the summer, is probably due to the fact that the organism cannot survive as well in ice as in water, and that it probably does not increase so rapidly in food or drinks, such as milk, during the cooler season.

Typhoid is a Disease of the Comparatively Young.—The greatest number of cases occur between the ages of 15 and 25 years of age and the numbers drop off sharply after the age of 45 years. It is more common among men than among women, probably because men travel more, eat all sorts of food, and drink all sorts of water.

The greater susceptibility of the young is believed to be due to the structure of the intestine.

Water as a Possible Source of Danger.—The organism cannot multiply in numbers to any extent in pure water but survives a very long time. The larger part of the organisms in water die off in 3 to 5 days, but 10 per cent may live 1 month, and 1 or 2 per cent for 1 month or 6 weeks. The typhoid organism does not form spores, so the form in which these survivors persist is unknown. As Whipple[18] points out, "It must be remembered that a single discharge of the bowels of a typhoid fever patient may contain one billion typhoid bacilli, perhaps more at times, and that 1 per cent of one billion is ten million, a number of bacilli large enough to do immense damage."

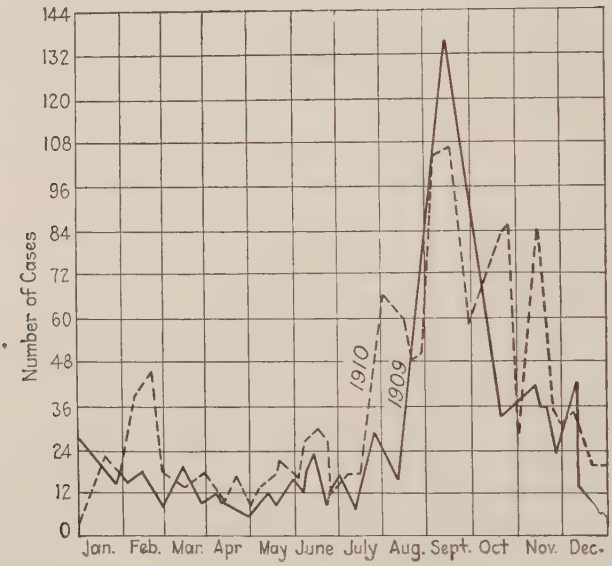

FIG. 130.—Typhoid fever is a seasonal disease.

Water.—In sewage, organisms do not survive as well as in pure or distilled water. The waters of the Chicago Drainage Canal carry the sewage of Chicago, including all the waste from the great packing houses, into the Illinois River and thence to the Mississippi. The inhabitants along these rivers objected to the presence of the sewage in these streams and claimed that there was constant danger of typhoid fever. Jordan, Russell, and Zeit,[6] in order to simulate conditions in the canal, suspended typhoid organisms in permeable colloidal sacks in sewage and found that they did not survive there nearly as long as in distilled water. The waste products of the sewage bacteria exerted a toxic effect on the typhoid organism which it escaped when suspended in pure water.

Bolton[1] was able to get similar organisms to live for months in distilled water apparently by the utilization of the dead bacterial bodies, the survivors becoming smaller and smaller in numbers, although after 7 months, 540 to 760 per cubic centimeter still survived.

Ice.—Freezing in water or in broth has little effect on bacteria if the freezing process is not continued too long. Intermittent freezing seems to be much more destructive than continuous freezing in killing *B. coli*. Very low temperatures play a minor part in bacterial killing, so Hilliard and Davis[5] suggest that the mechanical

operation of the crystallization of the water may crush many of the organisms, but after they are frozen, they are held in one position uninjured, regardless of the temperature to which the ice may sink.

Typhoid organisms, except 10 per cent, live only a day or two at freezing temperatures and only 1 per cent survive 2 or more weeks in a cake of ice.[13] These few survivors constitute the danger of freshly-cut ice which may have been contaminated by the sewage. In ice stored for 3 to 6 months, however, the organisms, even including these residual ones, should have died off and natural ice should be as safe as artificial ice.

If held in frozen ice cream, the typhoid organism has been known to persist for over 2 years, although all except about 2 per cent die off during the first 6 months.[12]

In Soil and Privy Vaults.—*B. typhosus* will persist for some weeks in the soil or in privy vaults, a maximum storage time of 52 days having been noted for the latter.[7]

Flies as Spreaders of Typhoid.—Typhoid fever is not contracted by being in the same room with a typhoid patient but may be transmitted by contact or by flies. The organisms are in abundance in the excreta and appear on the bed linen, the hands of the patient, or anything he has touched. All excreta should be disinfected by adding 5 per cent carbolic acid, allowing them to remain in contact for 8 to 12 hours before emptying, and linen should be washed in some disinfectant such as a 5 per cent solution of formaldehyde. The hands should be carefully washed after coming in contact with a typhoid patient and under no circumstances should anything in the way of utensils which have been in a typhoid sickroom be used for other persons before disinfection and washing. The sickroom should be screened to exclude the entrance of flies, as they may carry the organisms on their feet, and outdoor privies or toilets should be disinfected, especially if a typhoid epidemic is in progress.

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CHAPTER XXIX

BACILLUS DIPHTHERIÆ

(THE DIPHTHERIA BACILLUS)

(Corynebacterium diphtheriæ)

Diphtheria was one of the most dreaded diseases of childhood and early youth in former times, and this fear was well justified, for the percentage of deaths from it was usually from 30 to 50 per cent and in the Boston City Hospital before 1894, the mortality rate never went below 40 per cent. Eighty per cent of the deaths are below the age of ten years, and 17 per cent between the ages of five and ten years. The winter months, November, December, and January, always show the greatest prevalence. Now, 6 to 7 per cent of New York cases die.

The Occurrence of the Organism.—The organism of diphtheria occurs in the throat of the patient, from which it is quite easy to obtain and isolate in pure cultures. The organism was first isolated by Loeffler,[8] but as Klebs[7] had previously studied the organism in the throat, it is usually named the *Klebs-Loeffler bacillus*. It can be obtained by opening the mouth of the patient, depressing the tongue, and wiping the exudate (which is visible) from the back of the pharynx and from the tonsils. The swab, replaced in the culture tube (a sterile test tube), is sent to a laboratory where the examination is made. Or, if fresh medium is to be had, the swab is rubbed over the surface of a blood-serum slant without breaking the surface and the tube mailed or sent to a laboratory. The blood-serum culture is there incubated at 37°C. for about 12 hours, when it is ready for examination. The incubation gives the organisms obtained from the throat an opportunity to grow and they are now to be found in abundance in a smear made from the surface of the blood serum. The staining is usually done with methylene blue, as this staining brings out the characteristic granules. The stain originally used was suggested by Loeffler, the discoverer of the organism, but Ponder's stain brings out the granules more clearly and is in general use.

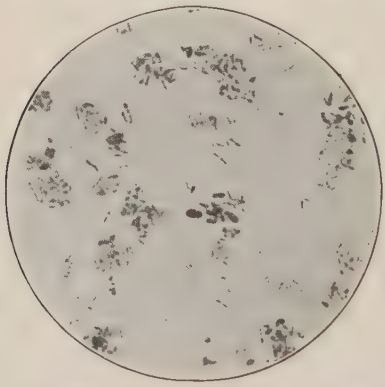

FIG. 131.—*Bacillus diphtheriæ*. (*From Kral Institute.*)

The Organism.—These bacteria are non-motile rods, 2 to 6 microns in length, and 0.5 to 0.8 micron in width. One or both ends are likely to be swollen and the body of the organism usually stains in granules or stripes (Fig. 131).

Klebs had seen and described this organism, but, since in many diseases a number of bacteria are associated, he was not sure whether a particular type alone caused the disease or acted only in combination with other forms, although, on inoculating the granular rods into animals, he found a peculiar sloughing of the tissues in the neighborhood of the inoculation. He was inclined to believe that diphtheria was of two types: one involved rod-shaped forms and the other, streptococci. Loeffler cleared up the situation by demonstrating that the streptococci were superficial in the membrane and were of the same type as those which were abundant in the mouth at all times, but that the rod-shaped bacterium was in the deeper layers and in great numbers in the old membranes. He was unable, however, to inoculate animals successfully with the organism to obtain a typical case of the disease, although he did grow pure cultures by using blood serum, planting organisms on the throat surface, not in its depths. The organism planted on the damaged mucous membranes of rabbits, guinea pigs, and pigeons develops typical diphtheritic patches. It never occurs in the blood vessels as a general systemic infection but is restricted to the neighborhood of the point of inoculation.

Alkalinity and the Growth of the Organism.—Many of the pathogenic bacteria grow most rapidly in an alkaline medium, while many saprophytes will thrive with a higher percentage of acid.[2,3] The medium in which the organism is usually found seems to determine this factor; blood has a pH between 7.30 and 7.50:[13]

	pH Limits	pH Optimum
B. diphtheriæ	6.0 to 8.3	7.3 to 7.6
B. coli communis	4.4 to 7.8	6.0 to 7.0
Ps. pyocyaneus	5.6 to 8.0	6.6 to 7.0
B. prodigiosus	5.0 to 8.0	6.0 to 7.0
Pneumonococcus	7.0 to 8.3	7.8
Staphylococcus aureus	5.6 to 8.1	7.2 to 7.6

The Membrane.—The membrane formed as a result of the bacterial invasion is characteristic of the disease and is used as one of the best clinical symptoms. This membrane covers the tonsils and back of the throat, sometimes to the posterior walls of the pharynx, about the third day after infection. It is of a dirty-white to yellow-white color and formed apparently by exudate from the mucous cells of this region. The membrane is dry on the surface but a softer layer lies next to the tissue. If removed, the surface is red, bleeding, and somewhat eroded and a new membrane soon forms to cover it. In some cases, a membrane may be formed in other parts of the respiratory system such as the nose and larynx. Associated with the formation of the membrane is a moderate fever of about 102 to 103°F. in ordinary typical cases. All throat infections are looked on with suspicion by the practicing physician and a bacteriological examination is made as soon as possible to determine the presence or absence of the Klebs-Loeffler organism.

TOXINS AND ANTITOXINS

The Toxin Formed under the Membrane.—The active work of the organism will be seen to be on the injured or weakened mucous membrane of the back of the throat where it has been able to get a foothold. The poisonous substances which it forms as it grows have a caustic effect on the superficial cells, stimulating the secretion of a lymph, containing fibrin, which hardens into the membrane. The leucocytes, which are always found in abundance wherever the body is invaded, also become

involved in the membrane. The bacteria find food in the exuding lymph and continue to multiply in numbers. The poisoning process keeps up, the poison is absorbed, and *general symptoms* appear from a *local source*. The bacterial invasion of all parts of the body is not necessary in order to induce a general constitutional derangement, since the poison is all that is required. A poison of this type is known to bacteriologists as a true *toxin*. Many organisms do not produce toxins but bring about pathological conditions in the body by other means, as noted on the anthrax organism.

Roux and Yersin[11] were able to show that the organism will develop its toxins not only when in contact with the mucous membrane but also in cultures in flasks of broth. As the older bacteriologists would have said, the organism can produce toxin *in vitro* as well as it can *in vivo*. They found, further, that inoculation of this toxin, after it had been freed from all bacteria by passing it through a Pasteur-Chamberland filter, would still induce the same symptoms as would the organisms themselves.

The production of toxin may be inside the bacterial cell itself, *endogenous*, or outside the cell in the medium, *exogenous*. In the diphtheria organism, it is secreted into the surrounding broth and is, therefore, exogenous, although possibly not so until the cells die.

The following possibilitie for toxin production have been suggested:

1. The bacteria may excrete the toxin as one of the normal products of growth in the same manner that animals throw out urea.

2. The bacteria may excrete the toxin as one of the abnormal products of the metabolism of amino acids supplied in too great abundance. It must be pointed out, however, that Uschinsky claimed to produce toxins from pure synthetic media and that Wagner, Meyer, and Dozier[16] obtained toxin from the botulism organism in any vegetable extract in which it would grow.

3. The toxin may be one of the products of the breaking up of the organism itself. The toxin in this case, therefore, would be strictly endogenous. This hypothesis is supported to some extent by experiments of Dernby and Walbum[4] with the diphtheria toxin. Toxin obtained from a very young culture of *B. diphtheriæ* is not very strong, possessing about 250 toxic units per cubic centimeter. If, however, this weak toxin is mixed with a solution of Witte's peptone and incubated for 20 hours, it will rise in strength to 600 units per cubic centimeter, or with peptone bouillon will increase to 1,000 units in 10 hours. The original toxin would seem, according to this experiment, to be an enzyme which continued to act in a suitable substratum, in this case, one of the peptones, and to increase in strength. The older cultures of the diphtheria organism are strong in toxins and, of course, it is in them that the organisms are dead and undergoing a degradation into intermediate products. These facts would suggest that the diphtheria toxin is endogenous and only becomes exogenous by the breaking up of the older cells. The general statement is made, however, that the diphtheria toxin is exogenous because it finds its way into culture media.

The discovery of toxins was one of the milestones in modern medical bacteriology, for it pointed the way to a means of *a cure by the products of the bacteria*. It was the beginning of the new science of *serology*. Behring had already developed tetanus toxin and antitoxin; but diphtheria, being a much wider-spread disease, the investigations on it attracted the attention of all the medical profession.

The Development of Antitoxin.—In 1894, Roux and Martin developed a substance to counteract the toxin. The organism is grown in pure cultures in flasks which have a

very broad base, so as to give a large surface exposed to the air. The broth becomes filled with the toxin after 2 weeks and the bacteria may be separated by means of a Chamberland filter. The nature of the toxin is not known, but it seems to behave in many ways like a special form of enzyme, although in some respects unlike usual enzymes. It may be destroyed by heat, as is an enzyme, although a much higher temperature is required. It is destructive to the cells which absorb it, acting as a poison to the heart muscles and to the nerves throughout the body in a case of diphtheria. Only a very slight infection of the throat is sufficient to poison the whole body and produce death. If some of this toxin is injected into an animal, the same effect is produced as by infection with the organisms. The toxin is probably produced inside the body of the organisms themselves from the materials which they absorb, if the work of Uschinsky,[12] who developed toxins from this organism when grown in a purely inorganic broth to which sugar and ammonium salts had been added as sources of carbon and nitrogen, is correct. The organism seemed to be able to synthesize toxin from these comparatively simple substances.

The Schick Test for Susceptibility to Diphtheria.—The Schick test is based on the reaction of the body to an injection of diphtheria toxin. If antitoxin is present in abundance in the blood, no reaction occurs, but if the individual is susceptible to diphtheria, a redness and then a browning and scaling occur around the point of inoculation.

Two cubic centimeters of a dilution of diphtheria toxin is inoculated under the skin of the arm. If the individual is susceptible to diphtheria, the region around the inoculation becomes red within 24 to 28 hours and remains red for 4 to 5 days, after which the red disappears and the skin develops a brown pigmentation with scaling of the surface layers.

Ehrlich's Chemical Theory of Immunity and Antitoxins.—Ehrlich[5] has suggested a theory of antitoxins, and of antibodies in general, which, as it covers many of the facts, has been quite generally accepted and used as a basis for further experimentation. It is usually called his *side-chain theory* because the basis for it, although probably only an analogy, is the benzene ring of Kekule with its accompanying side chains. The simple benzene is a six-sided figure with a CH group at each angle. It is possible to build up other compounds by the substitution of some other group for the H compound, so that very complicated structural formulæ may be constructed. The groups which lie off at the angles are the side chains and they may be very long and complex (Fig. 132).

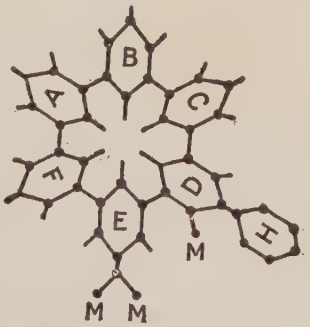

Fig. 132.—A biophore molecule, made up of peptones, *A* to *F*, and with unsatisfied valences at all points except *M* and *H*. (*From Adami.*)

Ehrlich regarded the living, or biogen, molecule as analagous to a benzene ring with a large number of long and complex side chains. Food must be brought chemically into some of the protein combinations by these side chains. It is hardly possible that the protein molecules which are taken into the alimentary canal, as meat or vegetable proteins, enter bodily, as such, directly into the building up of the body.

They are split up and are then attached to some preexisting molecules which are already serving some function in the cell. These molecules have unsatisfied valences which are completed by these additions from the outside. It is conceivable that some of the substances which are attached to the cell molecules are not, in all cases, of real advantage to the cell which includes them in its combination, but they have a valence which enables them to take hold and become a part of the group, although they may destroy the group as the result of such an attachment. The real food builds up these biogen molecular groups but poisons destroy them, or inhibit their proper functioning.

With these facts in mind we may have a clearer understanding of Ehrlich's conception of the structure and affinities of toxin and antitoxin. Much of his work was done on toxins other than those formed by the bacteria, but the general principles worked out are applicable to those of true bacterial origin. Ehrlich was able to show (1) that the reaction between toxin and antitoxin was not a vital one in which the presence of the human or animal body was necessary, for the reaction took place just as well in a test tube as in the body, although it was later found that the proportions varied if the experiments were conducted in a test tube, *in vitro*, as compared to their proportions if conducted in the body of an animal, *in vivo*. The reason for this difference is not known. (2) He was able to demonstrate that the toxin and antitoxin united to form a harmless compound, just as an acid and an alkali united to form a harmless (physiologically) salt. They did not destroy each other but united in a chemical combination which it was possible to break by heat, when the antitoxin may be destroyed and the original toxin remain. Or, the combination may be treated with weak hydrochloric acid and the antitoxin destroyed. In general, the toxin seems to be more resistant to heat and chemicals than the antibody itself and if one is destroyed, it is the antitoxin.

He suggests that toxin and antitoxin must be very weak since the reaction requires some hours to complete, as in mixing substances in very dilute solutions. The curve of the union of the toxin and antitoxin is a straight line similar to that which would appear if weak acid and alkaline solutions were mixed, but it must not be inferred from this analogy that he considered the toxin and antitoxin to be either acid or alkaline.

In order to present a graphic picture of the protoplasmic molecules with their receptive side chains and the toxins with their unsatisfied valences, he has given a concrete concept. These cell affinities are represented as projections from the molecules, each projection having a definite shape (Fig. 133). The toxin has one end fitting one of these points of attachments known as the *haptophore* (hapto = hold, phore = carrier) with the other end of different structure carrying the substance which is poisonous, the *toxophore* end of the toxin. The toxin

coming into contact with the molecule is drawn into it, because the configuration of the haptophore end will exactly fit the projection which the cell molecule offers, as a key will exactly fit the lock for which it is made. The lock and key analogy (Fischer) may be carried still further, since the toxin will not attach itself to any cell molecule, only those of a certain type. The tetanus toxin, for example, will not be attracted chemically by the cells of the foot but will pass unchanged to the central nervous system where it will meet side chains which fit it, and with which it will enter into chemical union to destroy the nerve cell.

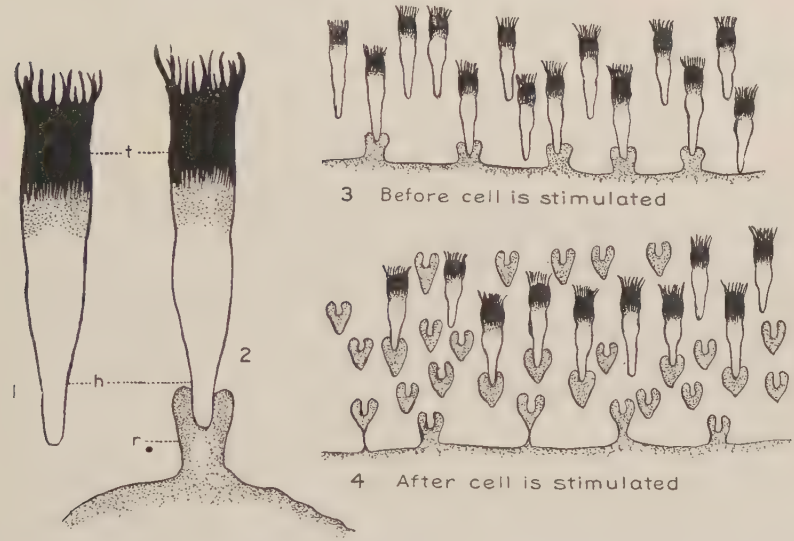

FIG. 133.—1, a toxin, consisting of *t* toxophore or poison bearers and *h* haptophore or attachment end; 2, a toxin attached to the receptor of a cell; 3 and 4, cell before and after stimulation to the production of an excess of antibodies. (1 *and* 2, *after Ehrlich; all diagrammatic.*)

The union of the living molecule with the haptophore end is without any results, but the haptophore, once attached, draws into the molecular structure the toxophore, which, in some manner, prevents the molecule, and the cell with which it belongs, from normal functioning.

The side chains produced are attached to the cell under normal conditions, at least nearly all of them, but if the cells are stimulated to the continued production of antitoxin by bacteria, or by toxin injection, part of the side chains will be attached, but many of them will free themselves from the biogen molecules to become distinct bodies entirely separate from the molecule from which they arose. These free side chains, known as free *receptors*, may unite with the toxin in exactly the same manner as do those which are attached to the biogen molecules the toxin uniting with them to form a harmless combination. The

object of antitoxin production, therefore, is to stimulate an overproduction of free receptors to unite with, and neutralize, the toxins, so that none of them may become attached to the cells.

This description of the toxin and antitoxin in diphtheria assumes that it is *unitarian*, that only one poison is produced by the bacteria and neutralized by the body. The problem is much more complicated than indicated, since secondary poisons, called *toxons*, by Ehrlich, are introduced by the bacteria, inducing some of the after effects, such as partial paralysis in the throat. Elaborate subsidiary hypotheses were suggested by Ehrlich to account for the neutralization of the toxons but it is unnecessary to discuss them in detail.

The Production of Diphtheria Antitoxin.—Owing to its poisonous nature, a very small dose of the toxin is sufficient to kill a small animal, such as a rabbit or guinea pig, so larger animals must be employed, the horse being the usual producer of the antitoxin. The horses used must be free from disease, such as anthrax or glanders. A little (only a fraction of a cubic centimeter) of the toxin from the cultures is injected into the blood of the horse, the introduction of the poison being followed by a rise in temperature and a temporary loss of appetite. As soon as the animal has recovered from the effects of the first dose, another is given, usually after an interval of a few days. This is repeated for 2 or 3 months when the animal is able to care for 300 cubic centimeters of the toxin, but in the beginning the injection of a fraction of a cubic centimeter would cause serious disturbances. Some chemical change has been going on in the cells of the horse that enables it to neutralize this large amount of toxin. They have been stimulated to produce an antibody which, uniting with the poison of the bacterium, renders it harmless. We cannot localize this production of antibodies; they may be secreted by the white blood cells, or by some of the other cells, or by all of the cells of the body, but they appear in the plasma of the blood. If some of the blood of the horse, taken from the jugular vein, is allowed to clot, the serum will contain this antibody in quantity. Five to eight liters may be drawn at a time without injury to the horse. This serum with its contained antibodies will keep for some time if preserved at low temperature. The strength of the antibodies in each cubic centimeter of serum varies with the animal and must be experimentally determined by inoculation. Since the antitoxin will neutralize the toxin in a test tube just as well as in an animal, a definite quantity of the toxin of a known strength can be mixed with a definite quantity of the unknown antitoxin and the combination inoculated into a rabbit or guinea pig, or the two may be inoculated simultaneously. If no reaction occurs, it may be accepted that the toxin has all been neutralized and, in the next trial, a larger amount of the toxin may be added to the same amount of antitoxin before it is injected, and so, by repeated trials, the real value of a cubic centimeter is determined. The antitoxin is put out in *units*. *A unit is the amount of antitoxin, which, injected into a guinea pig of* 250 *grams* (*about ½ pound*) *weight will neutralize one hundred times the minimum fatal dose of the toxin.* The dosage for a child is based partly on the body weight but the amount given is very variable, the urgency of the case and the judgment of the doctor determining the number of units to be given. For a child two years of age, 2,000 to 10,000; while for a child over twelve, 30,000 to 50,000 units are recommended. The serum is injected subcutaneously, intramuscularly, or directly into the veins, the latter method being used in cases where the need is urgent.

The introduction of the antitoxin into the blood stream of a patient suffering from a throat infection with the diphtheria organism releases at once into the blood an

immense number of these antibodies, which, uniting with the toxins produced by the bacteria in the throat, neutralize them and prevent their serious poisonous influence on the organs of the body. The organisms may continue to produce toxins but there are so many antibodies already in the blood that the effect is very small. It will be particularly noted that these antibodies do not affect the bacteria; all that they do is to care for the bacterial products, the destruction of the organisms must be left to the bactericidal bodies and to the phagocytes, and the latter soon free the body of the bacteria.

The remarkable effect of the antitoxin treatment is shown by the following figures (Rosenthal): of 183,256 cases treated in 150 cities previous to the serum period, *i.e.* before 1894, the mortality was 38.4 per cent; while since the introduction of serum, among 32,548 cases, there was mortality of 14.6 per cent, but if we omit those who died without the serum treatment, the mortality was only 9.8 per cent. It will be seen that the serum is not an infallible remedy but the fact that it has reduced the mortality to about one-fourth that of the preserum days shows the benefits from its use.

BACTERICIDAL BODIES

The organisms which invade the body are not only digested by the white corpuscles but are also injured by chemical bodies thrown off by the body cells which, when absorbed by the bacteria, weaken or kill them. These bodies are similar to those produced by the bacteria themselves; so, there is a constant exchange of fire of poisonous chemicals or toxins, similar to that which might occur between the artillery of two opposing armies. The bacteria are poisoning the animal cells by their discharges while the host cells respond by a fusillade of toxins injurious to the invaders, the *bactericidal bodies.*

If the bacterial cells have receptors to which these bodies will fit, they are absorbed, and the toxophore attacks the bacterial protoplasm. The body cells, however, cannot be expected to produce substances which will be toxic to all possible invaders so that the destruction of the bacteria in many diseases must be ascribed to other causes.

Criticisms of the Ehrlich Theories.—The Ehrlich theories, when first proposed, were subjected to much adverse criticism; some of it was very ironical of his airy and fantastic structure. He bolstered up his original suggestions by various accessory explanations and his system has been tacitly accepted and used for the practical purposes of immunology and this, too, in spite of the opinion of men like Jacques Loeb that serology is "a pseudo-science of juggled hypotheses and superficial analogies."

The basic conceptions of Ehrlich have been called into question recently by Manwaring[9,10] largely on physiological grounds. The methods used to obtain evidence as to the validity of the Ehrlich claims are too complex to describe in detail but, from the results obtained, the side-chain theory seems to be without any real physiological basis. An enzymatic theory is proposed by this investigator in its stead; the introduction of foreign proteins such as those which come from the bacteria stimulate the body cells to the secretion of enzymes by which the invaders are gradually broken up and destroyed. These simpler dispersal products may enter into combination with some of the proteins of the body and bring about specific reactions.

Two phases would, therefore, be passed in the introduction of foreign protein: its breaking up, and a possible synthesis of some of its products with the body proteins. Somewhat similar viewpoints are held by other noted pathologists.[1,14,15]

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CHAPTER XXX

MYCOBACTERIUM TUBERCULOSIS

(THE TUBERCULOSIS ORGANISM)

(*Bacillus Tuberculosis*)

Tuberculosis is one of the dreaded diseases of modern civilization. The savage tribes who live in the open air exposed to rain and cold do not succumb to this disease until the introduction of infection.

An estimate of the cause of deaths due to this one disease in the United States puts 10 per cent as due to this one infection, the life of one person in ten ends with this disease in some form. No race, sex, age, or country but pays its toll in deaths from tuberculosis.

The Organism.—The organism of tuberculosis, first isolated and studied by Koch[5], tested to the full the resourcefulness and persistence of this investigator. His isolation of the organism stands as one of the half dozen great achievements of bacteriology. Koch observed in tuberculous tissue peculiar, slender bacilli. He isolated them by cutting open one of the tubercles (the little nodules of tissue in the lungs or intestines which enclose the organisms) with sterile scissors and transferring some of the interior of the tuberculous material with a sterile platinum loop to the surface of a tube of solidified bovine blood serum. Growth is very slow, even on this very favorable medium, and from 10 days to 2 weeks are required at 37°C. to produce colonies; dry, whitish scales, which gradually fuse into a dry surface growth. The organism can be carried over to other serum cultures and may be continued indefinitely in the laboratory, although the growth is always very slow. Some investigators have used the yolk of egg alone; some have mixed the white and the yolk; and some have combined the white with other substances such as glycerine or meat broth. Growth is very good on any sterilized egg medium. Nocard and Roux,[6] in the Pasteur Institute, discovered that the tuberculosis organism would grow on ordinary nutrient agar to which 5 to 8 per cent of glycerine was added.

The Morphology of the Tuberculosis Organism.—The organism is a very slender rod, often bent, and internally broken up into lighter and darker staining areas (Fig. 134). The latter character induced some of the older observers to describe it as a spore former, but no spores have ever been observed, and the segments into which it breaks have never been seen to germinate. The granular appearance may be due to the deposition in the body of some of the staining material, as the cell is homogeneous with other methods.

The average weight of tuberculosis organisms is given as about 2.5 one-hundred millionths of a milligram, or there would be 40,000,000 organisms in 1 milligram of growth of artificial media.[2] The specific gravity of the organism varies between 1.01 and 1.08 so they fall to the bottom of liquid cultures.

On some media the organisms have a branching appearance (Fig. 135) similar to that of the fungi, so that it is evident that the slender bent rods are only one phase of

the organisms which appears in the stained sputum. This fact, pointed out by Coppen-Jones[3,4] gives the name *Mycobacterium tuberculosis*. Other peculiarities of *Myc. tuberculosis* make it a probable near relative of the Actinomyces group; possibly a degenerate species which readily disintegrates into rods of indefinite length. The

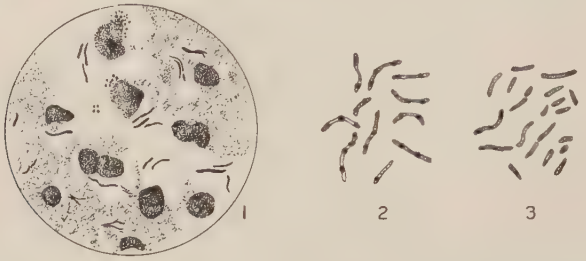

FIG. 134.—*Mycobacterium tuberculosis:* 1, the organisms as long slender rods, stained in sputum; 2, human tuberculosis rods; 3, bovine type.

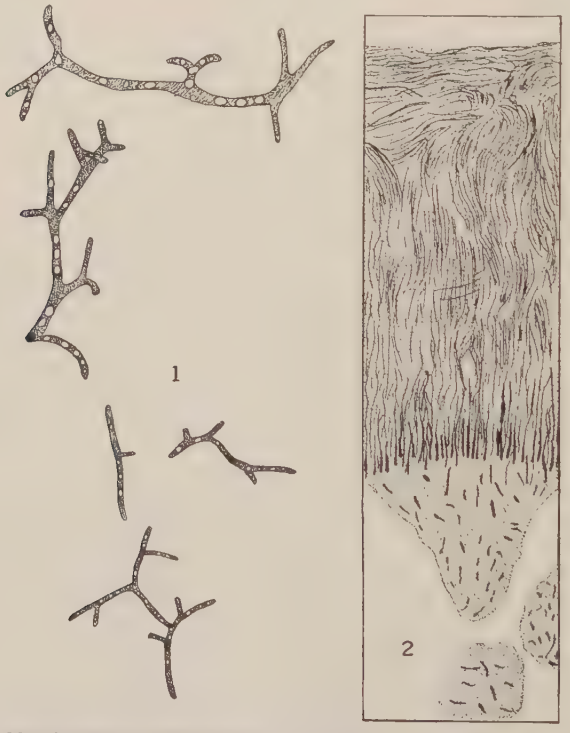

FIG. 135.—*Mycobacterium tuberculosis* in cultures: 1, branching forms; 2, section of a culture grown on agar showing the thread-like nature of the growth. (*After Coppen-Jones.*)

slow, dry growth, the formation of scale-like colonies, the branching, and the segmentation into fragments, all suggest this relationship. The organism is rather difficult to isolate from sputum as it is a very slow grower as compared to many of the types with which it is there associated, but modern technic has rendered this

possible; it can be readily stained in sputum in such a manner as to differentiate it from all other bacteria present in the lungs. The few organisms which have the same staining reaction are not likely to be found in the respiratory system, so that any bacteria giving a positive stain, according to the method known by the name of *Ziehl*, are likely to be tuberculosis organisms.

Staining Reactions.—The tuberculosis organism stains differently from most other bacteria, the wall being composed or impregnated with some substance which will not allow ordinary water-soluble aniline dyes to enter. Koch first noticed this fact in a negative way when he observed that all bacteria *except* the tuberculosis organism stain brown in an alkaline solution of methylene blue, this organism taking a bright blue color. Ziehl proposed the method, which, with modifications, is the basis of the present technic: staining the organism for 1 minute or more in a hot solution of the dye dissolved in carbolic acid (any of the anilin dyes used for bacteria may be dissolved in the carbolic acid but fuchsin has been decided to be the best, perhaps it gives a greater amount of contrast); the color is then removed by the use of weak acid in alcohol from all organisms except the tuberculosis bacterium. The other organisms may then be stained by a short immersion in some water-soluble anilin dye, such as methylene blue, and the field will show red tuberculosis organisms with other bacteria stained blue (Fig. 134). The wall and possibly some of the cell contents retain the color against the application of acids or acids in alcohol, *i.e.* it is *acid fast*, a very valuable characteristic in identifying it when mixed with other species. In addition to this staining reaction, the long, slender, sometimes curved, rods often show granules inside the cell, giving the organism a striped appearance with the usual technic. The cell wall is, to a large extent, the cause not only of the staining reaction but also of the very great resistance of this organism to death by desiccation, exposure to the action of weak disinfectants, or to the temperature of pasteurization.

Resistance of Mycobacterium Tuberculosis.—A temperature of 65°C. for 15 minutes, 70°C. for 10 minutes, or 80°C. for 5 minutes in liquids such as broth is required to kill it while the thermal death point of nearly all bacteria lies much lower, usually between 55 and 60°C. This means if milk is to be successfully pasteurized to destroy the tuberculosis organisms, the temperature must be kept at 70°C. for at least 10 minutes, and the scum which forms, if the bottle is kept open, may protect them so that they can resist a temperature of 60°C. for 1 hour.

This organism may remain alive enclosed in dried sputum for 6 to 8 months, although it usually dries up and dies in 10 days to 2 weeks, dependent to a considerable extent on the character of the sputum. Resistance to disinfectants is also marked as compared to the usual types and it may live for 24 hours in sputum in a 5 per cent carbolic acid solution.

The high degree of resistance to destructive agents is to be found (1) in the chemical composition of the cell wall which is impregnated by various fatty acids and some of the higher alcohols, which make it relatively impermeable to any substance in aqueous solution or to the entrance of water as steam, and (2) in the high osmotic pressure of the protoplasm which is unusually rich in the salts of phosphorus, calcium, and magnesium.

The organisms are very rich in albuminous and fatty substances, over 53 per cent of the former being present in thoroughly dried organisms and about 39 per cent of fatty substances.

The Fatty Substances in the Cell Walls.—If the fatty substance be removed by hot xylol after drying the organisms thoroughly in the oven, they then lose, to a considerable extent, their ability to hold the dyes against the action of acids, but the fatty substance obtained in the xylol will stain intensely in the carbol fuchsin and will be acid fast.[7]

Some investigators regard it as a non-saturated fatty acid, others as wax; and still others as one of the higher alcohols. Bullock and Macleod,[1] using boiling methyl alcohol, extracted the fatty substances from several kilos of the organism. When the alcohol cooled, a white precipitate was thrown down, the acid-fast substance, which saponified into fatty-acids and a snow-white powder, one of the higher alcohols, the latter the basis of the acid-fast property. The fat or wax, a secretion of the bacterium, is deposited to a large extent (95 per cent) in the wall of the organism, a little of it (5 per cent) in the protoplasm. It may render organisms sticky to each other.

The Production of Disease by the Organism.—Almost any organ of the body may be invaded by the organism, and while not usually found in the blood stream, it may travel partly in this manner. The lymph nodes are the organs particularly attacked, however, except in the ordinary pulmonary type. Small nodules or tubercles varying in size from microscopic granules to bodies as large as a mustard seed

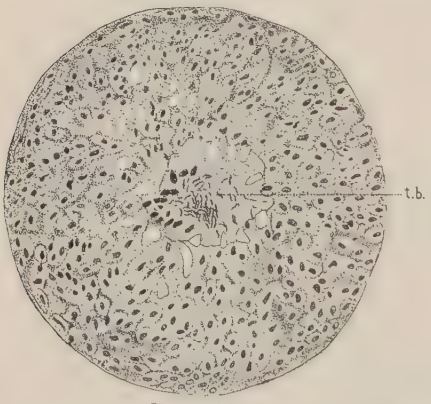

FIG. 136.

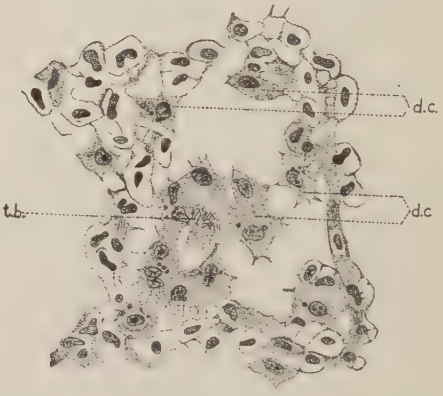

FIG. 137.

FIG. 136.—Miliary tuberculosis of the lung with giant cell and the tuberculosis bacteria *t. b.* imbedded in it. (*After Calmette.*)

FIG. 137.—Beginnings of a giant cell in the lungs (according to Borrel). The mass of tubercle bacilli in the middle *t. b.* are being enclosed by a mass of cells of phagocyte nature *d. c.*, to form one of the so-called "giant cells." These invading cells have been called "dust cells" by German pathologists. (*After Calmette.*)

are formed, these nodules being characteristic of the disease. The organisms are enclosed in these growths so that the tubercle represents the struggle on the part of the body of the person or animal to render these invaders harmless by shutting them into a cell or compartment.

The Formation of the Tubercles.—The formation of the tubercles is an interesting study in the pathology of animal tissues; the presence of bacteria leads to the formation of so-called "giant cells" in which the invading bacteria are imbedded, surrounded by a thick layer of epitheloid cells and undifferentiated tissue cells. There are two views as to the origin of the tubercle; the older one proposed by Baumgartner in 1885 has been upheld by Koch and many of the German pathologists; while the other explanation has received support from the entire Metchnikoff school.

Baumgartner claimed that, through the stimulation of the bacteria in the tissues, the epitheloid cells of the connective tissues of the lungs were induced to divide karyokinetically and fuse to form the large giant cells in which the organisms are held, as well as the epitheloid cells by which they are surrounded (Fig. 136). The

invasion of the lymphocytes is a secondary occurrence, since they only penetrate the tissues already formed.

On the other hand, the Metchnikoff school sees the formation of the giant cells and their surrounding tissue as the result of the invasion, of the region where the bacteria occur, by the uninuclear lymphocytes (Fig. 137). These cells fuse to form a single giant cell with many nuclei, sometimes as many as 60, which enclose the organisms. The work of these investigators on the formation of the giant cells in the lungs would seem to be fairly conclusive, since Baumgartner never claimed to have observed the division of the cells of the alveoli of the lungs except in certain cases and reasoned from the analogy of the formation in the eye and in the lung of primary giant cells.

The whole matter is still in dispute, however, and it is possible that they are not always formed in the same manner at all times. All are agreed fairly well as to the degeneration of the tubercles. The *caseation* of the tubercles is followed by the degeneration of the nuclei of the giant cells and the gradual destruction of stainable bacteria until apparently none remain, although in some cases an inoculation of old, degenerated tubercles into guinea pigs may produce the disease.

Different Types of Tubercle Organisms.—While the tuberculosis organism is usually associated with the human disease of that name, mammals, birds, reptiles, batrachians, and fishes are also attacked by strains of the same bacterium. The strains found in the cold-blooded animals or birds are different from those of the mammalia. The varieties which are commonest in the mammalia are known as the *human* and the *bovine types.*

The human type and the bovine type differ in cultures, both morphologically and physiologically. The bovine type is usually from 1 to 2 microns in length, while the human type is usually about 3 microns long; the human type too, is more likely to be slightly curved and fragmented (Fig. 134). In cultures, the bovine type grows well and can be isolated readily on non-glycerinated egg which has been coagulated, while the human type grows best on egg to which alkaline broth containing 5 per cent of glycerine has been added before being coagulated. The colonies which grow well on glycerinated egg, from fresh isolations, may be regarded as of the *human type,* while those that grow best on non-glycerined egg are of the *bovine* type. The human type is favored by acidity and the bovine by alkalinity. Other methods have been evolved which will futher differentiate the two types, so that it is possible to distinguish between them, even in cultures, without resorting to inoculations into rabbits.

On rabbits the bovine is much more malignant than the human type. Park inoculated 66 rabbits with 0.01 milligram of the bovine type and at the end of the sixteenth day all were dead except 6; while of 427 rabbits inoculated with 1 milligram of the human type, 235 were alive at the end of the sixteenth day and 175 of them had increased in weight. Even with an inoculum one hundred times larger, the human type was only about 50 per cent fatal.

Similar tests have been made on calves, a 50 milligram inoculum of the same organism suspended in physiological salt solution being injected under the skin. The *human* type forms large local abscesses but does not induce a generalized type of tuberculosis, while the *bovine* type causes a general internal infection. Eight cultures of the *bovine* type, but isolated from human beings, inoculated into eight animals in the manner described, caused generalized tuberculosis in six, resulting in death in from 23 to 63 days; one animal showed a general tuberculosis but did not die and the eighth remained healthy.

The general conclusions at which experimenters have arrived is that the *bovine* type is avirulent for humans and that the *human* type is avirulent for cattle. In spite of this lack of virulence of the bovine type, repeated investigations in Europe and America have led to the conclusion that a small percentage of the human cases are the result of the bovine type of organism. While the two types are not distinct,

intermediate varieties being found, the transformation of the bovine type into the human is a very difficult matter and the experimental evidence on it must be repeated in order to be convincing. In many countries the bovine type does not occur, and the human death rate does not seem to be much affected by its absence. Greenland and the islands of Guernsey and Jersey may be given as examples of such regions. A summary, made in 1911, of 1224 cases of human infection, collected in various countries, results in the following conclusion as to the type of organism involved:

Children under five years of age		Children between five and sixteen years		Adults over sixteen	
Human	Bovine	Human	Bovine	Human	Bovine
215	65	117	65	777	10

The percentage of bovine type is low in adults over sixteen years of age; much higher in children between the ages of five and sixteen years. Milk is one of the causes of the spread of the *bovine* type, so the infection of milk by tubercular cows is an important fact from the standpoint of infant feeding, where the dependence during the first five or six years is so largely upon cow's milk.

French estimates on infected cows show about 16.5 per cent reacting to the test for tuberculosis, the various provinces ranging from 10 to 30 per cent. About 25 per cent of the English cattle are affected; 30 to 35 per cent of the Italian; of German, 25 per cent; and of those in the United States, about 20 per cent. The infection of the udder of the cow is not absolutely necessary for pollution of the milk from tuberculous animals, since the tubercle bacillus has been found in 7 to 40 per cent of the cases where the dung was investigated. The organisms have been repeatedly found in the bile of infected animals so that they would be passed out in the feces. Dung often finds its way into the milk, so that the milk infection may occur in this manner. The fact that the drinking of milk often results in infection means that the entrance of the organism into the human body, especially in the case of children, is frequently through the intestinal walls.

Infection with the Tuberculosis Organism.—The manner in which the bacteria get through the walls has not been discovered but they are believed to enter and pass through the membrane without leaving the slightest trace of their passage. Once within the chyle-bearing vessels of the villi (the little out-pushings of the intestinal wall) they follow the lymph stream into the lymph nodes and may be carried to all parts of the body. Since the entrance of the bacilli is usually at a time when fat is being digested, the suggestion has been made that the fat droplets are ingested intact and that in them the tubercle organism is included.

The Poison Produced.—The statement that the organism does not produce toxin seems to depend on the meaning of the word *toxin*. If the toxin is a special poison excreted by the organism, which, when absorbed by the cells of the animal or human host, produces a reaction in the form of the development of antibodies to prevent that reaction, providing enough of them are produced in a free condition, then the tuberculosis organism does not produce a *true* toxin. If, however, the toxin theory is extended to include the reaction of the body cells to the introduction of any strange protein, then the tuberculosis organism does produce a toxin, but the toxin is largely an *endotoxin* derived from the bacterial protoplasm and only released on its disintegration. The inoculation of the bodies of dead bacteria under the skin of rabbits with the attraction of the leucocytes, or the inoculation of the

bacteria into the blood stream with the formation of young tubercles containing their bodies can only mean that the body of the animal responds in this way in an attempt to free itself from the bacterial protein.

The mere presence of the organisms is not sufficient to cause all the distressing symptoms of the disease: the wasting away, fever, loss of energy, inability to assimilate properly so as to overcome the loss from the disease, etc., in addition, of course, to the coughing which accompanies the pulmonary form. The bacteriologist can say only that the presence of the organism with its disintegration products produce in the body the reactions noted. The disease is usually due to a long-continued absorption of these products, and the cells of the body become so injured that they respond in the manner described. Tuberculosis is usually a disease of a weakened condition; but if the tubercle organisms with their weak virulence get started they begin the poisoning process. The remedy, therefore, is to make the body so healthy that it can wall off and destroy the invaders, and it is to this end that all modern methods of treatment tend.

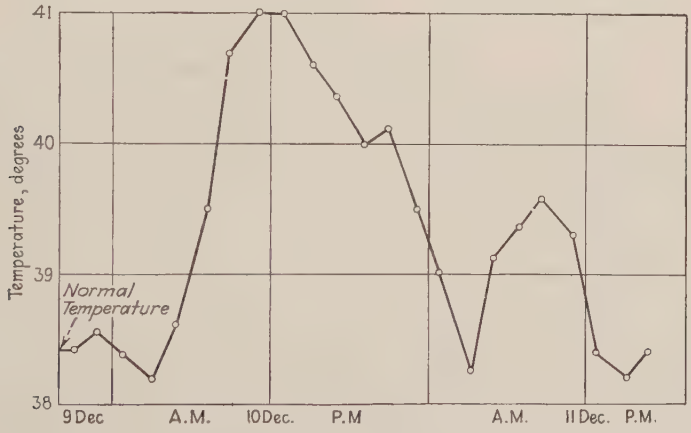

FIG. 138.—Typical tuberculin reaction in tuberculous cattle (*After Calmette.*)

Tuberculin.—The study of the effects of the inoculation of the dead bodies of the bacteria into animals led Robert Koch in 1891 to develop a preparation of the soluble portions of the dead organisms for the cure of the disease, his old *tuberculin*. Tuberculin was prepared from bacilli grown on glycerine agar. When a thick surface layer appeared it was scraped off into a 4 per cent solution of glycerine in water, the water evaporated until the solution had only one-tenth of the original volume, after which the bacterial remains were removed by filtration. The heating killed the bacteria, and the soluble portions of the endoproteins found their way into the surrounding solution of glycerine and water, warming over the water bath helping the rapidity of diffusion.

Various modifications of the original method of preparation have been made by Koch and other bacteriologists, the principal change being the use either of the products which the bacilli develop in a liquid medium, without including anything from the inside of the organisms themselves, or, the use of the ground-up bodies of the tuberculosis organism themselves, grown in culture, and mixed with glycerine as a preservative. The tuberculin prepared from cultures of the bovine type is *alkaline*, from the human type, *acid*. No explanation seems to be offered for this difference.

Koch suggested that the bacteria secrete into the tissues a substance which stimulates the necrosis of the tubercles. Inoculations with tuberculin show an increased focus, inflammation, and a necrosis of the margins; so while the tuberculin of Koch was developed as a possible cure for the disease, the fever which follows its inoculation, even in very small doses, with an aggravation of the disease instead of its alleviation, soon put an end to these hopes. It is, however, a valuable test of tuberculosis in animals and one of the important weapons of the veterinarian for the purpose. The normal temperature of the animal must be determined before the inoculation and for a day or two after the injection, to determine the effects (Fig. 138). If the animal has no tuberculosis, the injection produces no rise in temperature, but if there are infections, the temperature, after a slight fall for 5 or 6 hours, will begin to rise and attain a maximum about 24 hours after the injection. The temperature then drops gradually during the next 2 days and is back to normal in about 3 or 4 days. The injections, outside of the rise in temperature, seem to have very little effect on the general health of the animal. A rise of temperature of at least 0.8°C. is necessary to make an animal even suspected, and 1.4° rise is considered necessary to pronounce the test positive without a doubt.

Tuberculin is still used occasionally for the diagnosis of tuberculosis in human beings, although even small injections seem to produce focal reactions in the tubercles which persist even after the temperature has returned to normal. Subcutaneous inoculations seem to hasten the progress of the disease, producing a form of relapse from which the body has to recover, but other methods are more successful; the principal difficulty being that, as many individuals have been infected at some time, they react positively. It is occasionally used in some instances in the treatment of the disease; the effects vary, but in some cases the results are favorable.

The Cure of Tuberculosis.—The best and only treatment for the disease is plenty of good food, fresh air, rest, and no worry. The situation, as far as cures are concerned, has been summed up by Calmette as follows:

> Each one battles against the infection with his natural weapons, which are his leucocytes, his lymphatic glands, his cellular ferments, and his hereditary or acquired qualities of resistance or intolerance to the bacillus.

The danger to the individual comes from *mass* infections either through the intestine in the early years; or through any of the organs, but especially the lungs, in later maturity. The organisms are, at times, around us; we are often taking them in; we must get rid of them or succumb. Around us, at times, are human beings and animals who are eliminating the organisms and the problem of modern disease control is to reduce the dangers of infection to a minimum.

The organism of tuberculosis is interesting to the biologist as an example of the very low and slow form of pathogenicity. The organism secretes no particularly dangerous toxin, and only gets a foothold and advances in individuals susceptible in an unusual degree. The following sayings, quoted from Calmette, are to the point, "In tuberculosis, it is the soil which is everything and not the seed." "Tuberculosis is a diesase of poverty and ignorance."

The whole literature of tuberculosis has been summed up in the very admirable monograph, "Tubercle Bacillus Infection and Tuberculosis in Man and Animals," by A. Calmette, Associate Director of the Pasteur Institute, translated into English in 1923 and published by the Williams and Wilkins Co. of Baltimore. No extensive bibliography is really necessary, as it may be found in this volume.

An excellent American account of the organism can be found in "The Chemistry of Tuberculosis," by H. G. Wells, L. De Witt, and E. R. Long, 1923 published by the same company.[7]

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CHAPTER XXXI

SOME OF THE UNUSUAL BACTERIA

(COLOR- OR LIGHT-PRODUCING ORGANISMS, SLIME BACTERIA, AND HEAT-RESISTANT ORGANISMS)

Many of the organisms described are common and can be isolated in all parts of the world. A number of organisms exist, however, that are more difficult to find, which appear only rarely and then only can be isolated by special methods, while others are confined to certain regions of the world, such as the sea or to hot springs. Some are widespread but only in special habitats.

Some of the unusual types have already been described in more or less detail: the iron bacteria, the colored sulphur bacteria, the sulphur organisms, and the colored species of Staphylococcus; but some mention should be made of the following organisms:

1. The chromogenic bacteria which produce coloring matter in media.
2. The phosphorescent organisms which produce light.
3. The Myxobacteria or slime bacteria.
4. The thermophilic organisms which live and thrive at the temperature at which other organisms are killed.

BACILLUS PRODIGIOSUS

(The Red-Color-Producing Organism)

(*Serratia marcescens Bizio*)

While the red bacterium is one of the famous organisms of bacteriology, it is not at all common in the United States, except in bacteriological laboratories. It is called the "miracle organism" (*prodigiosus* = a prodigy or miracle) because, found at times during the past century on the host kept in Catholic churches, its colonies were believed to be drops of blood; it also occurred on various foods, such as the polenta used in Northern Italy. An investigation by various Italian scientists showed that it was due to a bacillus. Bizio called it "The phenomena of the red-colored polenta." Polenta is the corn mush used extensively as food in the northern part of Italy, and often kept for some time after making. The warm climate seemed to be adapted especially to the growth of this organism, abundant in air and dust, on the polenta. Bizio's name *Serratia* has been revived by the Breeds,[6] but the name by which it has become commonly known to bacteriologists is *Bacillus prodigiosus* or *Micrococcus prodigiosus*.[15,18,12,19]

The organism itself varies so much in form that it has been named both bacillus and coccus, the change being due, to a considerable extent, to the age of the cultures

examined,[9] rod-shaped types being characteristic of the young, and spherical forms of the older cultures.

The Coloring Matter and the Conditions of Its Production.—The coloring matter also varies from a faint pink to the deepest brown-red of dried blood, with one strain recently isolated of a rich crimson.[40] Since the coloring matter is the distinguishing character of this organism, the pigment has been the study of a number of bacteriologists; one of the first, Schroeter[41] separated the color from the organisms by placing cultures in alcohol, xylene, chloroform, and carbon disulphide, in any of which it was soluble, but insoluble in water. The coloring matter in *B. prodigiosus* is enclosed inside the cells and is never secreted to the outside, with the exception noted by Schreiner and Snow,[40] unless the organisms are dying, when it collects as granules between the cells, being insoluble in aqueous media. The individual organisms have only a very faint pink tint but in cultures on solid media may be a dark red, from the accumulated individuals.

The color production of a culture can be destroyed by incubating for a short time at blood temperature, as first pointed out by Schottelius[39] who placed a 2-day old culture, growing on potato, in an incubator held at a temperature of 38 to 39°C., for 24 hours. The color changed; the center was still a blood-violet but the margin of the colony, made up of new growth, became white. If two pieces of potato are inoculated with *B. prodigiosus* and one of them is left at room temperature while the other is placed in the blood-temperature incubator, the latter will be found to show only white colonies while the former has red ones. The color of the white colonies will return again if kept for 2 to 3 days at a cool temperature. If some of a culture which has been held for 10 to 15 generations at blood temperature is plated out, the colonies which develop at room temperature will have a red periphery with numerous white spots.

The coloring matter seems to be the product of the activities of the bacillus, since the young cultures show it only faintly and the red increases with age. Its formation may be prevented and growth still continue, so that it does not seem to be essential to vital activities.

Rosenberg[35] used the color, *prodigiosin*, as a test for cork and cutin, putting sections to be tested for 5 to 10 minutes in a solution composed of 5 grams of the bacterial substance in 25 to 30 cubic centimeters of 95 per cent alcohol.

Variability and Mutations.—The variability on alkaline media caused Beijerinck[4,5] to attempt to isolate from rapidly changing strains some which might be kept in pure cultures as permanent species which would not revert to the parent condition; such an organism would be in the deVries meaning, a true *mutant*. Beijerinck's plus and minus variants obtained from the parent strain seemed to be nearly as constant as the original mixed form. Wolf[49] attempted to induce the organism to vary from the normal by using modifications of temperature in which they were grown and by the addition of various poisons to the medium, but was unable to make any of them permanent; as soon as the culture was transferred back to the old medium and temperature, the modifications disappeared.

BACILLUS VIOLACEUS

(The Violet Bacillus)

(Chromobacterium violaceum, lividum, janthinum, etc.)

In fresh-water lakes, color-producing bacteria form one of the most numerous groups of water organisms, the most common color being yellow, followed in numbers by red, black, blue, green, fluorescent, and iridescent species.[42] Agar cultures made from water from natural sources often develop, if the cultures are kept for 1 to 2

weeks, white colonies which show a shade of violet or lavender, the tint deepening until the colony is a very dark violet, almost a violet-black.

The Organism.—The organism is usually a slender motile rod, 1 micron in breadth by 2 to 5 microns in length, with occasional short chains. A number of species of color-producing bacteria of this type occur and the organisms are very pleomorphic.[7]

The Coloring Matter.—The coloring matter is the important physiological character which is interesting, and much attention has been given to it by bacteriologists studying pigments. The pigment is produced either in the light or in the darkness,[11] although the shade is lighter and a little browner in colonies grown in the light.

Color production is dependent, as in *B. prodigiosus*, on the presence of magnesium and a phosphate in the culture medium; the other salts which favor its production apparently being those which serve to neutralize the injurious acids which arise during the growth of the organism.[43] Matruchot[25] has used the coloring matter extracted from cultures of these organisms as a stain for the living cells of one of the mucor molds.

THE PHOTOGENIC BACTERIA

The photogenic (photo = light, genic = originating) bacteria are a small but very interesting group of organisms, very widespread over the world, but not often observed unless special methods to induce them to develop and to isolate them are used. They had been known, long before bacteriologsts studied them, as the shining spots which glowed in the darkness on meat or fish that had been salted.

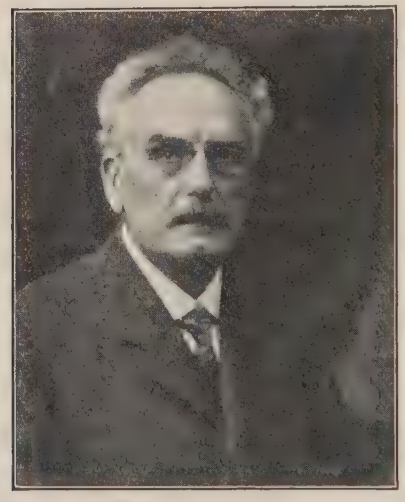

Fig. 139.—Hans Molisch, investigator of the iron and photogenic bacteria.

The best method for producing them is to take a piece of meat, the size of the fist, place it in a glass dish of sufficient size that it may be about half covered with a 3 per cent solution of common salt in tap water. This is covered with a bell jar and allowed to remain for 1 to 3 days at a rather low temperature (9 to 12°C.) when it will be found to have developed shining spots on its surface. Molisch,[27] (Fig 139) who recommends the above method, has found that, in Vienna, he could obtain colonies of the photogenic organisms on 89 per cent of beef tested and on 65.5 per cent of the horse flesh. At the time when the glow is first noted, the odor of decaying meat is very faint, and, in fact, the glow is the first indication of its spoilage. If decay of the meat goes on, however, the putrefying organisms overgrow the photogenic type. From the glowing star-like colonies, pure cultures may be isolated (Fig. 140), probably of the organism first studied by Cohn, renamed by Molisch, *Bact. phosphoreum*. On the other hand, if fish are used, the colonies are so abundant that one author has claimed that all fish glow after being kept. This glowing, however, is not an indication that they are unfit for food, as phosphorescence appears only before real putrefaction has set in.[28,29]

The production of bacterial light in pure cultures has been studied with reference to salt, an indispensable addition to a medium upon which they will grow and phosphoresce. The medium used for the purpose of isolation must contain some sodium chloride, the best amount being usually considered to be about 3 per cent, approxi-

mately that of sea water. MacKenney[24] found no growth on media which contained only 0.25 per cent and slight growth with 0.5, 10, and 15 per cent. The range lies, therefore, for maximum growth between 1 and 5 per cent. These organisms seem to have adapted themselves to a medium very rich in salts. Molisch[27] found that chlorides of sodium, magnesium, potassium, and calcium allow the production of light and also growth, vigorous growth being usually associated with light production.

The light bacteria found in solutions of sodium chloride led Gerretsen[11] to study the effect of both sodium and chlorine on the amount of light. Chlorine can be replaced by other ions without seriously affecting light production but the rôle of sodium can be filled only by magnesium without a serious loss of light; magnesium chloride will give about the same amount of light as sodium chloride. Any soluble sodium salt causes light production and the hexose sugars increase it.

The production of light in the ordinary varieties is favored by rather low temperatures but, in the tropics, photogenic organisms have been described which grow best at a temperature of 28 to 38°C.

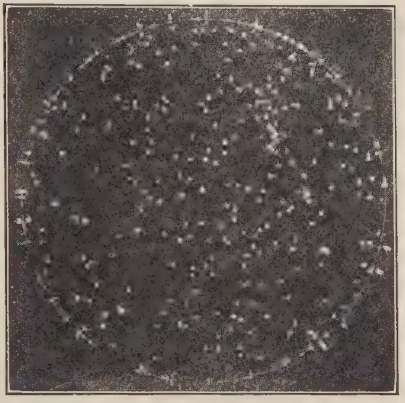

FIG. 140.—Photograph of *Micrococcus phosphoreus* taken by its own light. Colonies 8 days old; exposure 15 seconds. (*From Molisch.*)

Oxygen Necessary for Light Production.—The phosphorescent bacteria will grow only if oxygen is present, as Beijerinck[4] has shown in the very interesting experiment where he mixed photogenic organisms with 10 per cent gelatin in sea water and introduced, at the same time, sea diatoms or other algæ which would produce oxygen in sunlight, or diffused daylight. This gelatin placed between parallel glass plates, in order to exclude the air, was exposed to the light. The algæ form oxygen in the

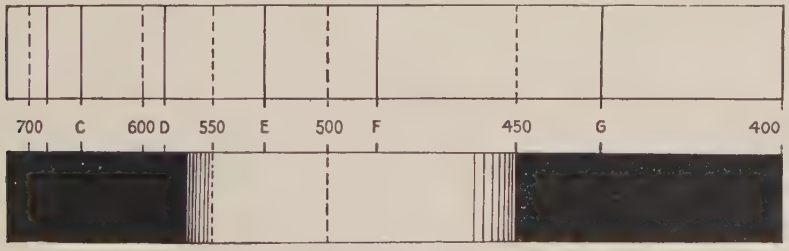

FIG. 141.—The spectrum of *Bacterium phosphoreum* is continuous between 570 and 450. (*After Molisch.*)

gelatin, but only the colonies of photogenic organisms in their vicinity glow in the dark. Some carbon dioxide must have been included in the gelatin for the assimilation of the green algæ or the total exclusion of both carbon dioxide and oxygen would result not only in anærobic conditions but also in a stopping of all photosynthetic activity by the algæ. The amount of oxygen, however, need be only a trace; 0.005 millimeter pressure is sufficient to produce light;[16] or they can derive oxygen from KNO_3 if it is used in the medium instead of salt.[35]

The light appears to the eye as a gentle glow of a yellowish to greenish tint with a continuous spectrum unlike the brilliant, but intermittent, light produced by such

insects as the firefly (Fig. 141). The relation of phosphorescence to light and electricity is complex; the recent literature from the standpoint of a physical chemist can be found in Allen.[1] At the Paris Exposition in 1900, a hall in the Optical Palace was lighted by using glass vessels whose inner surface had been coated with gelatin inoculated with light-producing organisms. The amount of light given off is very small, however, and the room must have appeared almost dark to visitors, since the light power of an area of culture estimated at 2,000 square meters is necessary to produce as much as comes from a single candle of standard size. Harvey[17] tried to measure the energy used in oxidation by the luminous bacteria and computed that they are not efficient power plants for producing light.

This light, although it has a very low candle power, seems to be very active in its effect on the photographic plate. Colonies on a Petri dish will impress their image on the photographic plate in an exposure of only a few seconds, and Molisch[27] succeeded in photographing objects by the light furnished by cultures with an exposure of 15 hours. This light will also affect seedlings, such as those of the pea, which are positively heliotropic, causing them to bend, if they are placed in the dark room with a culture of *Bact. phosphoreum*, in a positive direction (Fig. 142).

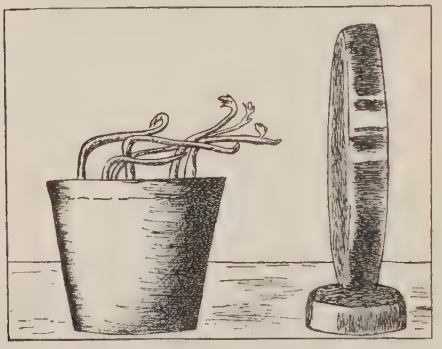

Fig. 142.—The light produced by the luminescent bacteria affect the growth of bean seedlings. (*After Molisch.*)

The cause of the light is much in dispute. Ludwig[23] claims that the bacteria secrete a substance which lies outside the cells and produces the light extracellularly. This substance, an organic chemical of an indetermined composition, is believed to be an aldehyde. The other viewpoint as to the origin of the light (Beijerinck)[4] held by the majority of investigators who have studied this group, is that the light is simply one of the expressions of the life functions similar to respiration or enzyme formation and results from the destruction of peptone. As Molisch[27] points out, however, there are many observed facts that are not in accord with such a theory, among which may be mentioned the fact that the light may be at its brightest when the growth is at a minimum, as a result of the low temperatures. The process is entirely intracellular but the substance which produces the light, and which has been called *photogen*, never passes out of the cell but remains there and functions in somewhat the same manner as an intracellular enzyme like zymase.[14]

By the use of ultra-violet light the organisms can be killed without stopping the production of light which seems to be a chemical process, the result of a light-producing substance, *photogen*, acted upon by an oxidase enzyme intracellularly produced, *photogenase*, or *luciferase*.

In the Light Organs of Marine Animals.—While the photo-organisms are very abundant in all sea water and occur occasionally in fresh water, one of the most interesting habitats, from a biological standpoint, is some of the sea animals, especially squids, which live in the warmer waters at a depth of 600 to 5,000 feet. In some of these animals the bacteria are confined to certain glands, but in others they are found in closed "light organs" scattered over the whole body surface.

Each variety of squid has its own typical light-producing bacterium with which it seems to live in a symbiotic condition.[26] These organisms have adapted themselves to a life in the serum of a particular species of animal and are not found in that of any

of the related cuttle fish, nor are they closely related to the phosphorescent species found in the sea water. The life of the organisms in these light organs is apparently a symbiotic one, although the advantage to the cuttle fish, unless the small amount of light produced is a benefit, is not very evident.

THE MYXOBACTERIACEÆ (SLIME BACTERIA)

One of the most puzzling and interesting groups of bacteria, from the standpoint of the student of the life history of the bacteria proper and the biologist who interests himself in cell aggregates and the possible forms of such aggregates, is this group of almost unclassifiable organisms with some of the habits of the slime molds and the morphology of bacteria. They were first studied by Thaxter[45] who found them growing on decaying wood, fungi, and lichens, being attracted to them by their resemblance to the fruiting condition of slime molds. He cultivated them in the laboratory and discovered that, in nature, a number of species occurred which varied both as to the form of the fruiting body and the bacteria which compose them. These organisms seem to be very widespread, having been since described, not only from North America but from various parts of Europe, Africa, Java, and Japan.

The life history of one of the rod-shaped forms of the species, *Chondromyces crocatus* (Fig. 143), is composed of two distinct phases:

1. A vegetative period in which the organisms continue vegetative growth for an indefinite period of time, dividing in a manner similar to the ordinary bacteria to form irregular colonies which radiate out in strands of individuals. The individuals are motile but not as ordinary bacteria, by active swimming, but by a slow gliding motion with an accompanying bending which sometimes may double the organisms over, the movement being only comparable to that of Beggiatoa or some of the blue-green algæ. As with the latter organisms, the slime on the exterior of the cell wall plays some rôle. The organisms are held together by slime in a mass similar to the plasmodium of the slime molds, but no fusion of individuals occurs, each cell retaining its identity. During this period the wet organisms are held in a liquid condition, but some internal metamorphosis seems to occur in the plasmodial mass.

2. The resting or sporulating period starts as the colony continues to increase in size; the individuals at the center begin to accumulate and to heap up into irregular masses. The bacterial mass may divide and branches develop, the differentiation going on by the formation of branches of the second, third, and fourth order (Fig. 143 Nos. 2, 3, 4). The organisms moving upward to form this complicated structure secrete, in this species, an internal stalk called, by Thaxter, a "cystophore." The cystophore is composed of the gelatinous secretion of the bacteria as they glide upward, mixed with the bodies of some of the stragglers left behind in the progress. The stalk is sometimes twisted, as shown in No. 3, but a straight stalk and branches, as in Nos. 2 and 4, is the more usual condition.

The plasmodial mass of the last branch collects at a number of places on the swollen end (Nos. 3 and 4) to form little papillæ. The bacteria gather finally into an egg-shaped mass, secrete a gelatinous envelope around themselves, and form a cyst (No. 5). The cysts drop off readily from the branch upon which they were formed and are distributed like the conidia of a mold.

Germination of the Cysts.—Under favorable conditions they germinate, a swarm of bacteria coming out of them and the vegetative cycle is repeated (No. 7). The

bacteria, in the cysts, are slightly curved rods with walls not very different from vegetative individuals; germination in this species consists in the bursting open of the common cyst wall.

Cultivation of the Organisms.—These organisms grow on sterilized dung, decaying leaves or other vegetation, but do not thrive on ordinary nutrient media. They may be grown readily on agar in which the nutrient substances are extracts from rabbit dung, or a 2 per cent malt extract.[22] The life history of many of the species described by Thaxter[46] and Yoshii[50] was derived from cultures grown on agar.

The *vegetative bacteria* show no flagella,[48] so that movement must be due to the external slime layer.

FIG. 143.—*Chondromyces crocatus:* 1, the bacteria collecting as a mass which is rising above the substratum, young cystophore at *b*; 2, cystophore developing a stalk, the mass of bacteria dividing into three branches; 3, nearly mature cystophores with branching of the third and fourth order; 4, immature cysts; 5 and 6, mature cysts; 7, mature cysts, one germinating; 8, rodshaped bacteria emerging from a cyst; 9, *Myxococcus coraloides*, one of the forms assumed by this species; 10, the bacteria as vegetative rods; 11, mature coccoid spores from a cyst. (*After Thaxter.*)

The above description applies only to the species described; other species vary in the formation of the resting spores, in the shape of the fruiting bodies, and of the cysts. The following classification is from Jahn:[20]

A. In the fruiting stage, the rods shorten in varying amounts but do not round up.

1. The plasmodium forms no rounded cysts, but heaps up into irregular masses or grows out into finger-like or columnar masses.
   1. Family: *Archangiaceæ*
2. The plasmodium forms cysts of a definite shape.
   a. The cysts are angular and small.
      2. Family: *Sorangiaceæ*.
   b. The cysts are round.
      3. Family: *Polyangiaceæ*.

B. The rods shortened into round spores.

4. Family: *Myxococcaceæ.*

Disputed Phases of the Life Cycle.—Many points in the life history and physiology are in dispute. The details of the formation of the cysts are somewhat different according to Quehl,[34] who claims that the whole mass rises upward on the cystophore, owing to the drying out of the aggregate at the base, and that the drying-out process continues until the organisms are forced out to final branches, where they dry as cysts. Thaxter's observation of a column of the bacteria climbing the cystophore, after it was completed, had led him to the conclusion, previously given, that the cysts are formed from later-ascending masses of bacteria. Vahle[48] is inclined to take Thaxter's viewpoint, as he has observed strands or columns of bacteria moving upward with the middle of the strand left behind as the cystophore.

The change from vegetative to resting condition has never been explained. The suggestion of Baur[3] that the formation of fruiting masses results because the myxobacteroids were forced out of the substratum upon which they are growing, the development of other organisms in the medium making it unfavorable for their growth, cannot be correct since they form the same type of fructifications when grown in pure cultures on agar.[46,47,48] In the vegetative condition, they are adapted to a moist substratum but if the substratum dries on its surface, vegetative growth must be checked. The drying of the substratum with an accumulation of excretion products would seem to be a tenable suggestion for the change in the mode of life.

The life of these organisms is usually saprophytic on the dung of herbivorous animals, such as that of the rabbit, deer, etc., and on decaying vegetation,[22] but Pinoy[31] hass uggested that the bacteria composing *C. crocatus* must be grown in association with one of micrococci. He[31,32] also believes that the legume bacteria belong to the group of the Myxobacteria; the swarming inside the cells of the legume being homologous to the plasmodial stage. Nixon[30] has shown cyst-like stages in the life history of the pear-blight organism. Geitler[13] describes a Myxobacterium, parasitic on the alga, Cladophora, which enters by dissolving the wall to make a hole just large enough to squeeze through and absorbs all the cell contents, nucleus, chromatophores, pyrenoids, and starch; the whole process requiring about 42 hours.

The relationship of the group to other lower organisms is very puzzling. The rod or coccus shape, retained in many species through life, would make them bacteria while the formation of a pseudoplasmodium gives them slime-mold affiliations. Thaxter pointed out in his original paper, however, that, in the Acrasiæ, the identity of the amœba-like individuals is never lost by the formation of a true plasmodium. Pinoy's suggestion that the legume nodule organisms are related, or are members of this group, indicates another species where the individuals retain their individuality. The method of reproduction of the Myxobacteriaceæ is very similar to that of other members of the Schizophyceæ, so that, on the whole, they would seem to be a special group of bacteria.

THE THERMOPHILIC AND THERMODURIC MICROORGANISMS

The thermophilic and thermoduric bacteria form a small group of species and strains whose protoplasm is adapted to a temperature which would kill ordinary types. The name "thermophilic" (thermo = heat, philic = loving) implies that these bacteria, algæ, and molds which thrive in such high temperatures as 70 to 80°C. in a habitat of hot springs, geysers, and waters in the tropics, do so from choice and not from necessity. The more recent work and review of the literature (Robertson,[36,37]) suggests that these organisms are forced by their surroundings to take on new

properties, such as the ability to survive high temperatures, on account of some undetermined properties of their protoplasm or cell wall; that is, they have become *thermoduric* (thermo = heat, duric = resisting).

The term "thermoduric," first used by Eckford,[10] has been adopted by Robertson as best describing them. The presence, therefore, of such bacteria or algæ in thermal springs is an indication that some individuals, able to survive such unusual conditions, give rise to a new physiological race. The original discoverers considered that they were a separate and distinct species and many were named because of their peculiar habitats, but the majority seem to be simply resistant strains of ordinary species.

Since these organisms are favored by a high temperature, maximum growth is attained by raising them in an incubator at a temperature of 55°C., as seen[44] in

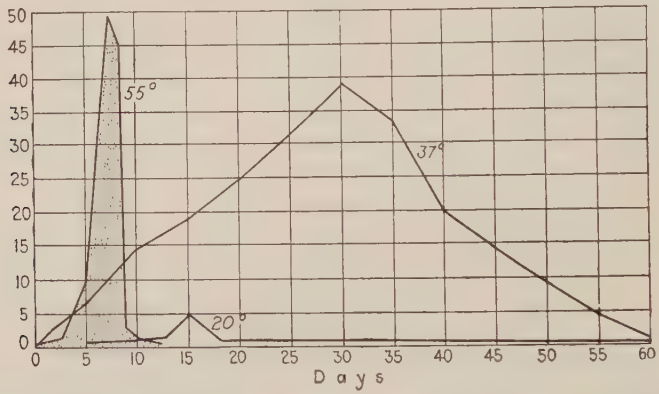

Fig. 144.—Growth of typical thermophile at various temperatures. The growth is largest and most rapid at 55°C., although nearly as much will occur in longer time at 37°. (*After Tanner and Wallace.*)

Fig. 144. While growth at 37°C. approximates the amount attained by the higher temperature, the 55°C. shortens the lag period and a maximum is attained in 5 days against 25 to 30 days required for 37°C. The growth curve of this organism should be compared with that of *B. carotovorus*, a bacillus adapted to ordinary soil temperatures, on page 238.

The Importance of These Thermoduric Organisms.—The first observers who noticed these living cells in water not many degrees below the boiling point regarded them as biological curiosities and attached scarcely any significance to them.

Recent work has been stimulated by the importance of the thermoduric types in canning and pasteurizing, since many anomalous results can be explained by the presence of bacteria which do not succumb to the usual sterilization temperatures. The spore-bearing types are themselves thermoduric; a small, visible body, the spore, serving to carry the organism through unfavorable surroundings, but many thermodurics are not spore bearers and give no visible morphological evidence of their peculiar physiological resistance to heat. The older statement, that pasteurization rendered milk practically sterile except for spore

formers, must be modified by recent work. A very small percentage of non-spore bearers escape pasteurization, these bacteria being principally of the lactic-acid forming group.

The method of isolation of such organisms is simple. A sample of water, soil, milk, feces, or silage mixed with a nutrient solution is kept at an abnormally high temperature, 62°C., for 7 hours. Only the organisms or strains which can live and grow under such conditions are to be found at the end of this time. They may be plated out and obtained in pure cultures, incubating the plates at the same temperatures, the minute colonies formed being known to the dairy bacteriologist as the *pin-point flora* of pasteurized milk.

Various authors have reported the isolation by this or similar methods, of streptococci, micrococci, sarcina, and non-spore-bearing rods from pasteurized and boiled milk. Ayers and Johnson[2] identified a thermophilic species, *Lactobacillus thermophilus*, to which they attributed the property of growing at pasteurizing temperatures. Robertson,[36] from 197 cultures isolated from milk from various sources, identified five thermoduric species: a streptococcus, a lactobacillus, a micrococcus, and two sarcin æ. Growth did not appear at the pasteurizing temperature, but ove 90 per cent of the cells survived.

The Cause of the Thermal Resistance.—The cause of the resistance is not known, but Robertson was able to kill young cultures of three of the commonest and most resistant organisms which he found in milk, *Microbacterium lacticum*, *Sarcina lutea*, and *Streptococcus thermophilus*, much more easily than old cultures where the older cells had passed the accelerative growth stage (see p. 174).

The addition of sugar to the culture media increased the number of individuals which survived high temperatures; with *Streptococcus thermophilus*, in a broth which contained 1 per cent of sugar, 53 to 326 escaped pasteurization at 66°C. for 30 minutes, but with 41.2 per cent sugar from 2,000 to 15,000 of the same cultures still lived. Sucrose increases the osmotic pressure in the media and tends to dehydrate the protoplasm, resulting in a lower moisture content and, consequently, a higher coagulation point. Associated with this protective action of the sugar was a pronounced capsule which may have rendered difficult the passage of water from outside into the cell protoplasm. Bacterial death is probably due to coagulation of the proteins, a process retarded by a dehydrated protoplasm.[21]

The group of thermoduric organisms are most interesting to the biologist, since they may be associated later with resistance to other conditions such as cold, desiccation, and chemical action. As has already been noted, a small percentage of bacteria survive long periods in frozen condition in ice (*B. coli*, p. 203); some bacteria withstand many days of desiccation, although the majority succumb quickly; and a small number remain after the application of disinfectants if not in too concentrated a solution nor for too long a time. The question arises, whether all of these forms of resistance are not the same phenomenon and that it is the explanation for the survival in the soil and dust of many varieties of non-spore bearers. In these "duric" types, the organism may be on the way to the formation of spores or have developed a substitute for them. No comparisons have been made between strains which show resistance to heat, cold, chemicals, and desiccation, but such studies would be interesting, as they might explain these biologically aberrant individuals.

Spore-bearing Thermophiles.—In addition to the organisms which have no spores but thrive at high temperatures, a series of bacteria forming spores, apparently derived from the soil, which will grow well at 50°C. in milk[33] and in canned vegetables and meats,[8] have been described. Some of these bacteria are strains of common varieties such as *B. subtilis*, while others are apparently new species.

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CHAPTER XXXII

THE KILLING OF BACTERIA BY DESICCATION, LIGHT, HEAT, PRESSURE, AND CHEMICALS

Bacteria in nature die from the effects of desiccation (drying), light, and lack of food. Man kills bacteria by the use of two of the same physical agents, desiccation and light, and also by the use of heat and chemicals.

DESICCATION

The ordinary non-spore-bearing species will withstand desiccation for some days. Careful studies made on many disease-producing varieties give results which vary with almost every organism and surrounding condition. The diphtheria bacillus, for example, may live 194 days in ordinary air, while tuberculosis germs enclosed in thick sputum may survive months, although the time which most organisms survive is much shorter. Combined with desiccation is always associated lack of food; the life processes of oxidation and respiration continue at a slow rate, but in the course of time the organisms succumb from exhaustion of the food.

LIGHT

Light has been known to injure bacteria since the pioneer work of Downes and Blunt[13,14] showed that direct sunlight would stop growth, diffuse light check it, and that blue and violet rays were more deadly than red or orange, while heat rays did

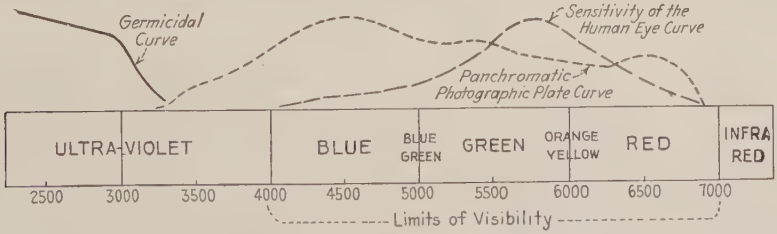

FIG. 145.—The spectrum of white light with the regions affecting the eye, a panchromatic photographic plate, and bacteria indicated by curves.

not affect them at all. The ultra-violet rays are even more destructive,[3] and are the efficient part of white light (Fig. 145). Since such rays do not pass in quantity through ordinary glass, however, much of the effect of the sunlight is lost in glass-enclosed rooms. Ultra-violet light passes through quartz, so that if any large proportion of this constituent of sunlight is to be utilized, glass must be replaced by quartz window panes. In open sunlight, the full effect of the ultra-violet light is obtained.

Special lamps, such as the Cooper-Hewitt mercury-vapor lamp, used in photography and for special commercial lighting, supplies a high percentage of ultra-violet

light, and can be used for killing bacteria. The sterilization of water and the pasteurization of milk by such lamps have been attempted. Ultra-violet rays penetrate well into water, so that they have been successfully used for the sterilization of water for drinking purposes.[18] The water is run in a thin sheet under a battery of mercury vapor lamps which give off sufficient light of this type to destroy about 98 per cent of the organisms, the process being regarded as efficient and cheap as filtration. Similar experiments made[2] on the pasteurization of milk were less successful. When the rays were applied in such a manner that the numbers of bacteria were reduced to a satisfactory number, the milk acquired a disagreeable flavor which made it unsalable. The thickness of the layer exposed, and the length of time of the exposure, were the two factors which controlled the reduction in numbers, the best method evolved making use of a revolving drum which carried the milk under a battery of lamps.

Sunlight as a Germicide.—Sunlight undoubtedly destroys a large proportion of the bacteria in nature, since an exposure of only a few hours is necessary to kill the

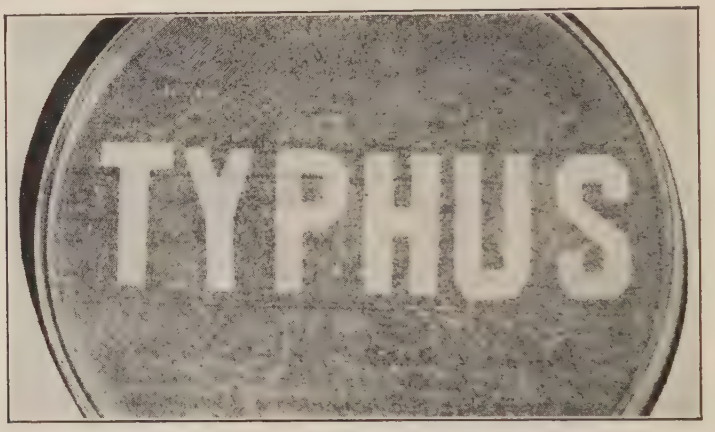

FIG. 146.—A heavily seeded plate of *B. typhosus* after exposure to sunlight, but protected under the word "typhus" by black paper letters. (*Buchner.*)

majority of the non-spore bearing types. Buchner[7] demonstrated this fact by placing a large number of the typhus bacillus in agar and pouring the agar into an ordinary Petri dish. After it had hardened, part of the bottom of the dish was covered by letters, cut out of black paper and fastened to the glass. The dish, exposed bottom side up for 5 hours to diffuse daylight, was then brought back into the culture incubator. After 2 days of incubation, the plate was covered with colonies in the region where the black paper had protected them (Fig. 146).

Cause of This Action of Light.—Such a destruction of the bacteria might be effected either by changes in the culture medium due to the exposure to the sunlight or by changes in the bacteria themselves. Marshall Ward[37] was not able to grow the typhus bacillus as well on gelatin which had been exposed for 2 to 3 hours to direct sunlight, and believed that the light induced chemical changes in the media, detrimental to bacterial growth.

The present viewpoint on the action both of sunlight and of the ultra-violet rays is that they coagulate the protoplasm in an irreversible reaction and that the organism or cell dies. The action of ultra-violet light on egg albumen is to change the isoelectric point.[9,10]

Proteins absorb the ultra-violet light and have a well-marked band in the ultra-violet region, the amino acid, tyrosine, being the special cause of this absorption,

although phenylanine has a similar band.[17] If organisms are placed in solutions of gelatin, peptone, tyrosine, or leucine, they are protected from ultra-violet rays, the tyrosine being especially protective and detoxicating. Or the protective action may be confined to some of the nuclear derivatives, such as cytosine, thymine, or uracil. [15]

The germicidal wave lengths lie between 350$\mu\mu$ and 185.6$\mu\mu$ with the shortest ones the most lethal to the bacteria. An increase in hydrogen-ion concentration or in temperature makes the short rays still more effective, but neither temperature nor acidity will make the long rays germicidal.[6,5,4,11,24] Spores are especially difficult to kill, 2 to 8 hours in sunlight being necessary, sixty times as long as for the vegetative bacteria.[38] The ultra-violet rays constitute only about 1 per cent of the total energy in sunlight; the amount of ultra-violet is at a maximum about noon and much more falls during the summer than in winter;[19] much of it is lost in the smoke which hangs over northern cities such as London.[20]

HEAT

The sterilization of materials and apparatus both in the laboratory and in hospitals is effected by the use of heat in the form of dry air, steam, or steam under pressure. The use of chemicals leaves an objectionable residue in the media while sterilization by filtration through a Pasteur or Berkefeld filter is only applicable to small quantities of liquid media.

Hot air maintained at a temperature of 160°C. for 1 to 1½ hours is usually sufficient to destroy both the bacteria and their spores. At this temperature cotton plugs will be slightly browned from the heat and the bodies of the bacteria are also probably slightly charred in spite of their low water content.

Steam works much better and will destroy both bacteria and spores if applied at least 4 hours. The majority of the vegetative bacteria will not stand a temperature of over 80°C. for 1 minute. The yeasts and molds are killed almost instantly in steam and mold spores are nearly all destroyed by the temperature of pasteurization.[33]

Tyndall worked out a method by which the spores of the bacteria which withstand steaming for 1 hour uninjured can still be killed. The principle consisted in the application of 1 hour of steaming on 3 successive days, the intervening time giving the spores an opportunity to germinate. This method of *intermittent*, *discontinuous*, or *fractional* destruction of the bacteria has been widely used for sterilizing gelatin and blood serum, and has certain advantages if the tubes or flasks are not strongly infected with spore-bearing types. To be successful, the glassware in which the medium is to be placed must be sterilized, since the water from the surface of solid media will creep up along the sides of the tubes, and spores from the tube will germinate in it even after the medium itself is sterile.

The sterilization of blood serum is effected by a process in which the heat is not allowed to rise much above 85°C., the medium being kept in a slanting position in tubes.

Discontinuous sterilization has proved to be too slow, and canning is now carried out by a 3 to 4 hour steaming. Miquel and Lattraye[27] have shown, that, even if placed in a nutritive medium, a few of the spores do not germinate for many days, and escape the 3-day steaming.

Steam under Pressure.—The only alternative to the use of flowing steam (at approximately 100°C.) for 3 to 4 successive days is the application of steam under pressure. Steam under pressure can be raised to a higher temperature than 100°C.

and is so much more effective that the intermittent method is no longer extensively used, the addition of 5 to 10° of temperature to flowing steam at 100°C. being all that is necessary to complete the sterilization in an hour. Steam under pressure is usually calculated as pounds per square inch; the relation of the pressure to temperature is as follows:

Pounds pressure	Temperature degrees Fahrenheit	Degrees Centigrade
0	212	100
1	215	102
5	227	108
10	239	115
15	250	121
20	259	126

Miquel and Lattraye, by the use for only 15 minutes of 110°C. (approximately 6 pounds pressure) for 3 successive days sterilized substances where flowing steam for 1 hour for the same time did not kill all the spores.

One objection to very high temperatures is that they caramelize the sugars in the media.

The age of spores and the medium in which they are placed has much to do with the time required for germination. Some of the phenomena of spore formation and germination have already been discussed under *B. subtilis*, page 175; *B. mycoides* (p. 187), and *B. anthracis* (p. 254). The extreme resistance found in a small percentage of the spores would suggest the possibility that the bodies found in the bacteria are of at least two types: (1) an easily germinated and killed variety and (2) a slow germinating and very resistant form.

Effect of Heat.—Heat coagulates protoplasm but the smaller the percentage of moisture the higher the temperature to induce coagulation.[26] Dry warm air, for this reason, must be at a higher temperature than moist warm air or steam. The protoplasm of the bacteria or the bacterial spores absorb moisture when surrounded by steam and so are more easily coagulated; but death from the coagulation of the cell proteins, because of the small size of the organisms, makes any direct observations impossible.

PRESSURE

The Effect of Very High Pressures on Bacteria.—Bacteria are probably adapted to an atmospheric pressure, such as found on the surface of the world, *i.e.*, about 15 pounds to the square inch, but under conditions, such as in the depths of the ocean at a depth of 3,500 feet, where pressure must be enormous, sea-slime bacteria may still be found.[31,31a]

Hite, Giddings, and Weakley[21] hoped to kill all organisms in canned foods by short exposures to extreme pressure. The heating, ordinarily used for sterilization, always changes the taste and chemical composition of preserved material; both food value and flavor are valuable. The commercial aspect of such preservation was found to be impractical because of the enormous pressures (40,000 to 120,000 pounds to the square inch) which must be used to destroy even non-spore-forming types.

The curve developed in the death of *S. lactis* (Fig. 147) is fairly typical of those obtained from similar organisms tested, such as *B. prodigiosus*, *S. lactis*, *B. fluorescens liquefaciens*, and ordinary yeast. The results obtained were not at all in agreement, but they are interesting as a proof of the relative stability of the organisms even under such enormous strains suddenly applied.

The pressures were obtained by means of a hydraulic press and a specially prepared tube of steel built up in a manner similar to that used in the construction of very large cannon, one jacket of steel being heated and then shrunk over the next and smaller inner section. A single, case steel tube had been broken by a pressure of 130,000 pounds pressure to the square inch, but this built-up tube withstood pressures up to 180,000 pounds without disaster.

The question naturally arises as to the effect of such pressure applied suddenly on proteins. Cider subjected 90,000 to 120,000 pounds pressure for 120 to 130 minutes, when kept for a year, showed all the pulp thrown out of the solution and precipitated

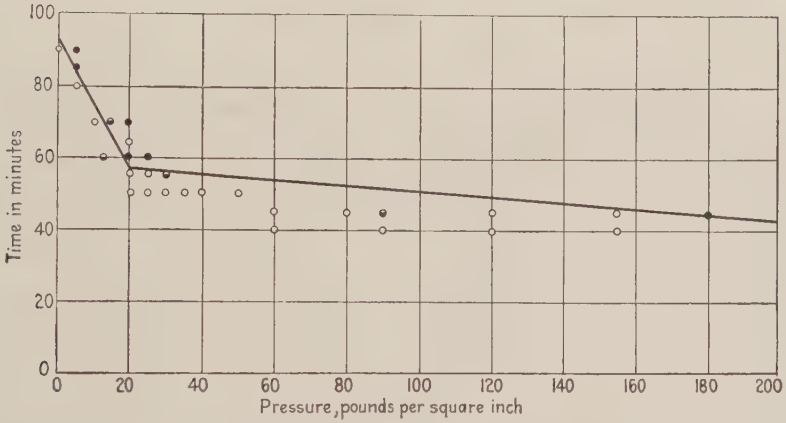

FIG. 147.—Effect of very high pressures on lactic acid organism (*S. lactis*). Cultures above line were in general killed; those below line survived. The death of organisms was a function of time and pressure. (*After Hite, Giddings, and Weakley.*)

as a lining to the vessel in which the experiment was performed, the liquid in the center being absolutely clear.

Roger[30] subjected various organisms, among which were *B. coli* and *B. anthracis*, to pressures of 2,000 and 3,000 atmospheres (30,000 and 45,000 pounds) to the square inch for 2 minutes. The pressure rose for 10 minutes, was held constant for 2 minutes and then was suddenly dropped to normal. While saprophytic organisms were uninjured, the streptococcus of erysipelas showed retarded growth but marked increase in its virulence; on the other hand, the virulence of the spore-free *B. anthracis* was so lowered that, if held at 3,000 atmospheres, the culture would kill guinea pigs only after 18 to 19 days, and at 2,000 atmospheres in 12 to 13 days.

Even higher pressures were used by Larson, Hartzell, and Diehl.[25] A direct pressure of 6,000 atmospheres killed non-spore-bearing forms in 14 hours but 12,000 atmospheres were required to kill spore bearers in the same time. In carbon dioxide, non-spore-bearers were killed by 50 atmospheres in about 1½ hours but the death of the organisms was not due to the acidity produced but to the gas itself.

The death of the organisms might have been due to coagulation or to internal ruptures resulting from sudden changes, inside and outside

the body, just as the internal organs of deep sea fish are sometimes everted when these animals are suddenly brought to the surface, but no experimental evidence has been offered on morphological changes in the bacteria.

ANTISEPTICS, GERMICIDES, AND DISINFECTANTS

The Killing of Bacteria by Chemicals.—The chemicals used against bacteria may have one of two purposes: either to suppress growth as an *antiseptic* or *bacteriostatic* agent, or to destroy them as a *germicide* or *disinfectant*. The same chemical may be either an antiseptic or a germicide, depending on the strength used.

The antiseptic action of copper is shown in Fig. 148, the organism in the lighter portion of the plate being *B. carotovorus*. The copper prevents growth near the dime, a few individual bacteria appear in the next zone outward, and beyond this are unaffected organisms. Some organisms might show a zone of stimulation where the copper is in the correct proportion to increase cell division; this phenomenon does not appear in the *B. carotovorus*.

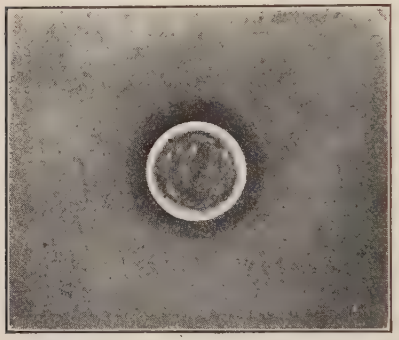

FIG. 148.—Scoured dime in a heavily seeded plate of *B. carotovorus*. Its 10 per cent of copper prevents growth near the coin; a few resistant organisms grow in the next zone; and outside, the bacteria form normal colonies.

The fact that bacteria are checked in their growth or are killed by chemical action has been known almost as long as the organisms themselves, but the publication in 1865 of Lemaire's book on "Acide Phenique" (carbolic acid) marked an epoch which seemed to Keen,[22] who was a student in Paris at the time, comparable only with the notable carbolic-acid disinfection era ushered in by Lister a few years later. Davaine[12] was soon testing out the length of time necessary to kill the organism of anthrax, and Schroeter,[32] working in the prepure culture days under the influence of Cohn, tried out a number of different chemicals that were in favor at that time for disinfection purposes, among which were potassium permanganate, chlorine, and, especially, carbolic acid. The proportions in which he used these chemicals were much weaker than now found advisable, the object of his experiments being not to kill the bacteria but to prevent their growth. For this purpose, 1 part of carbolic acid in 1,000 parts of water was a concentration in which no organism could continue growth. A weaker solution would hinder growth; such action being only of an *antiseptic* nature and not necessarily germicidal. Schroeter, however, points out the fundamental fact, which later work confirmed, that the effect of a disinfectant must be determined in the laboratory on the particular organism for which it is intended, since there is much variation in the response of different bacteria to chemicals. Koch[23] made elaborate tests both on vegetative *B. anthracis* and on its spores dried on threads to determine their resistance to carbolic acid. Lister had used 5 per cent carbolic acid as a part of his surgical dressings and inaugurated modern antiseptic surgery. The chemical in general use for a long time was 5 per cent solution of carbolic acid; all

other disinfectants were compared to it. The consequence was that this concentration of carbolic acid has become the standard solution, and for many reasons (particularly cheapness), it is still one of the best.

Numerous other substances have been tried but chemists and bacteriologists are still seeking the ideal disinfectant and antiseptic. Two apparently impossible conditions are to be met: (1) the ideal disinfectant or antiseptic must destroy bacteria in a reasonably high dilution; (2) it must be physiologically harmless to the person or animal to which it is applied, *i.e.* it must be non-poisonous. The latter condition is absolutely essential if the antiseptic is to be applied to wounds, since the healing of wounds is brought about by the growth of adjacent cells. Unfortunately, all known chemicals which prevent the growth of bacterial cells have somewhat the same action on human or animal cells as they do on bacteria, although some are less irritating in their action than others.

In the use of substances as germicides, especially carbolic acid, which has been most carefully studied, two factors are involved (providing the temperature is constant): (1) the strength of the disinfectant and (2) the length of time which it acts. In many of the older experiments the carbolic acid was left for hours or even days in contact with the culture to be killed. As soon, however, as these two factors came to be recognized, the time was shortened to a few minutes, and the concentration of the disinfectant increased. The strength of the solution in length of time of its application may be expressed in the form of an equation as follows:

$$\frac{-Dt}{Dc} = \text{Constant}$$

in which t is the time, c is the concentration and D is a variable dependent on the organism and chemical. If the time is lengthened, the concentration may be lessened to obtain the same constant; on the other hand, a decrease in concentration would have to be compensated by a lengthened time.

Various laboratory methods have been devised to determine these factors as accurately as possible. In the Rideal-Walker[29] methods a 12-hour old broth culture of *B. typhosus* is placed in the various strengths of disinfectants to be tested. At the end of 2½ minutes, transfers are made, from each of the concentrations used, into fresh tubes of broth, and similar transfers at the end of 5 minutes, 7½ minutes, and 15 minutes. If the *B. typhosus* cells have all been killed, no growth results in the tubes to which they have been transferred.

The original Rideal-Walker method for determining the value of a disinfectant has been further improved in the U. S. Hygienic Laboratory.[1,28] The details are slightly different, but the basic principles are essentially the same. Pure, standard phenol is used instead of commercial carbolic acid, as it has been found that many commercial brands of the latter vary in their percentage of phenol. The tubes in which the tests are made must have very little organic matter in solution, as it has been found by several investigators[8] that many disinfectants, such as corrosive sublimate, combine with organic matter present in the culture medium and gradually weaken.

The determination of the value of a disinfectant is important to the buyer and seller of disinfectants, although it is difficult to compare two very unlike chemicals acting very differently on bacteria. The method consists in general in making a series of dilutions of the disinfectants to be tested, exposing the organism to this series for 2½, 5, 7½, 10, 12½, and 15 minutes, and making transfers to sterile tubes of broth. A similar set of dilutions of phenol is tested at the same time with the same organism. At the end of the incubation period, the two sets of tubes are examined. The weakest dilution of the disinfectant in question that will kill in 2½ minutes is compared with the weakest dilution of phenol that will kill in the same time. The same is done with the weakest solution that will kill in 15 minutes. The average is taken of the

two, as is shown in the table given below, the value of the disinfectant in terms of phenol coefficient being derived from the average of the two figures.

EXAMPLE ILLUSTRATING THE HYGIENIC LABORATORY METHOD OF PHENOL COEFFICIENT

Disinfectant	Dilution	Time in minutes					
		2½	5	7½	10	12	15
Phenol	1–80	−	−	−	−	−	−
	1–90	+	−	−	−	−	−
	1–100	+	+	+	+	+	−
	1–110	+	+	+	+	+	+
Cresol	1–80	−	−	−	−	−	−
	1–160	−	−	−	−	−	−
	1–200	+	+	−	−	−	−
	1–225	+	+	+	+	+	−
	1–250	+	+	+	+	+	+

From the 2½-minute exposure $\frac{160}{80} = 2$

From the 15-minute exposure $\frac{225}{100} = 2.25$

The phenol coefficient is 2.1.

The phenol coefficients of many common disinfectants now on the market have been determined in this manner so that it is usually possible to know, from the label on a disinfectant, its comparative value. Many of the commercial disinfectants are phenol or cresol preparations and are nearly all within the coefficient range of 1.0 to 5.0. Some of them are practically as effective in the presence as in the absence of organic substances, but the majority have their efficiency cut down by any form of complex organic material. Tests for the use of the disinfectant in the presence of organic matter are the same as those described, except that the different disinfectant dilutions (5 cubic centimeters) contain 2 per cent of peptone and 1 per cent gelatin. The results obtained by McClintic with some of the commercial disinfectants was as follows:

Disinfectant	With organic matter	Without organic matter
Bacterol	1.34	1.58
Carbolene	0.65	1.36
Chloro-naptholeum	3.21	6.06
Creolin-Pearson	2.52	3.25
Cresoleum	1.75	2.90
Crude carbolic acid	2.63	2.75
Germol	1.79	2.12
Kreosota	0.65	1.26
Lysol	1.57	2.12
Saponified cresol	0.57	1.03
Trikresol	2.50	2.62
Zonol	1.57	2.37

Elaborate tables have been prepared showing the relative values of various disinfectants, but they are of more or less doubtful value as the particular use to which each chemical is put modifies its efficiency. Some very useful disinfectants may not have a very high phenol coefficient. Such a disinfectant is lysol, in which the active agent is cresol combined with soap in such a way as to render it quite soluble and particularly effective with bacteria which are protected against many disinfectants by their own wax or fat-like material. On the other hand, corrosive sublimate, with a very high coefficient, is much weakened in the presence of protein, since it will combine as well with the protein as it will with bacteria themselves. Some disinfectants will not penetrate well, while others, such as alcohol, penetrate rapidly. The standardization of methods for testing disinfectants has been further improved in this country by Reddish[28,24] by using a series of organisms: *B. typhosus*, *M. aureus*, *B. diphtheriæ*, *B. tuberculosis*, and the *Pneumonococcus* instead of the usual single organism, *B. typhosus*. A phenol coefficient is obtained for each of these types, a fairer approximation of the value of the disinfectant being obtained in this way than from trials on a single organism, since organisms differ in their susceptibility to disinfectants and a compound which will destroy one will have little effect on another, even if used in the same percentage.

SUMMARY OF COMMONLY USED DISINFECTANTS

1. Salts of the Heavy Metals.—Mercuric iodide and chloride, among the most powerful germicides known, are violent poisons and combine with the proteins and other complex organic matter; silver iodide and nitrate, about as strong, are also highly poisonous. Copper sulphate is weaker.

2. The Halogens.—Iodine, bromine, and chlorine are all strong disinfectants as are many of their combinations, although not all. The chlorine is much more effective when freshly released.

3. Organic Compounds.—Alcohol is a weak disinfectant unless used in a 50 to 95 per cent solution when it kills largely by plasmolysis. Phenol (carbolic acid) and cresol are among the best cheap organic germicides known, either used alone or mixed in soaps. Formaldehyde is a gas, highly soluble in water, much used in aqueous dilutions, and also as a gas for disinfecting rooms after cases of contagious diseases.

4. Oxidizing Compounds.—Hydrogen peroxide, potassium permanganate, and sulphur in the form of SO_2 are all germicides which have been used, although the peroxide and SO_2 are not as useful as was once believed. Miquel has listed the chemicals which have some antiseptic value in the form of the table, the results of a long series of tests which he made. The table is probably not entirely correct but indicates, to some extent at least, the dilutions which are necessary to prevent growth but not to kill.

The Best Antiseptics	Proportion
Mercuric iodide	1 in 40,000
Silver iodide	1 in 33,000
Hydrogen peroxide	1 in 20,000
Mercuric chloride	1 in 14,300
Silver nitrate	1 in 12,300

Very Strong Antiseptics	Proportion
Osmic acid	1 in 6,666
Chromic acid	1 in 5,000
Chlorine	1 in 4,000

Very Strong Antiseptics	Proportion
Iodine	1 in 4,000
Chloride of gold	1 in 4,000
Bichloride of platinum	1 in 3,333
Hydrocyanic acid	1 in 2,500
Bromine	1 in 1,666
Copper chloride	1 in 1,423
Thymol	1 in 1,340
Copper sulphate	1 in 1,111
Salicylic acid	1 in 1,000

Strong Antiseptics	Proportion
Benzoic acid	1 in 909
Potassium bichromate	1 in 909
Potassium cyanide	1 in 909
Aluminum chloride	1 in 714
Ammonia	1 in 714
Zinc chloride	1 in 526
Mineral acids	1 in 500–333
Thymic acid	1 in 500
Lead chloride	1 in 500
Cobalt nitrate	1 in 476
Nickel sulphate	1 in 400
Uranium nitrate	1 in 356
Carbolic acid	1 in 333
Potassium permanganate	1 in 285
Lead nitrate	1 in 277
Alum	1 in 222
Tannin	1 in 207

Moderate Antiseptics	Proportion
Quinine bromhydrate	1 in 182
Arsenious acid	1 in 166
Boracic acid	1 in 143
Strychnine (sulphate)	1 in 143
Sodium arsenite	1 in 111
Chloral hydrate	1 in 108
Sodium salycilate	1 in 100
Ferrous sulphate	1 in 90
Caustic soda	1 in 56

Feeble Antiseptics	Proportion
Manganese perchloride	1 in 40
Calcium chloride	1 in 25
Sodium borate	1 in 14
Morphine (chloride)	1 in 13
Strontium chloride	1 in 12
Lithium chloride	1 in 11
Barium chloride	1 in 10
Alcohol	1 in 0

Very Feeble Antiseptics	Proportion
Ammonium chloride	1 in 9
Potassium arsenite	1 in 8
Potassium iodide	1 in 7
Sodium chloride	1 in 6
Glycerine	1 in 4
Ammonium sulphate	1 in 4
Sodium hypophosphite	1 in 3

SOME COMMON DISINFECTANTS

Phenols and Cresols.—A large percentage of the commercial disinfectants on the market are phenols and cresols, since, in them, two essentials are combined, efficiency and cheapness. For the same amount of money, more disinfectant (as measured by its efficiency) can be obtained than by the purchase of any other compound. The cresols are about two and one-half times as strong, weight for weight, as the phenols, the commercial cresol being usually a mixture of all three cresol compounds; cresol forms three compounds, all with the same chemical radicals but arranged differently inside the molecule and all differing slightly in their properties.

Iodine.—Iodine and the other halogens, chlorine and bromine, are all very active disinfecting agents. Iodine is usually used in a strong alcoholic solution and is very irritating, but not so markedly as some others. Chlorine is usually used as a gas, and is especially germicidal when freshly released.

Formaldehyde.—This compound, introduced under the trade name of "formalin" is a gas (usually about 37 per cent) dissolved in water. It is used as a liquid, when diluted ten to forty times, for all kinds of disinfection. As a gas, it has been used very extensively for disinfecting rooms after cases of contagious diseases.

Mercuro-chrome.—This compound of mercury and chromium is one of the newer and more popular disinfectants, especially for small local infections where the solution must be applied to living tissue.

Soaps.—Soaps help to remove the bacteria, mechanically, by freeing them from the body in washing, and are, in themselves, something of disinfectants, even in a pure condition, against certain susceptible bacteria such as the pneumonococcus, staphylococci, streptococci, and the diphtheria bacillus. In strong lather, soap will kill these organisms in 1 minute,[35] probably by lowering the surface tension of the bacterial cells. The toxic effect seems to vary, however, with the organisms to be killed and with the soap used. Some organisms, such as *B. subtilis* and *B. anthracis* in a vegetative condition, are more easily destroyed than others, such as *Ps. aeruginosa or B. prodigiosus.*[16] Soaps made from coconut oil are much more effective than those made from linseed oil, the difference being apparently due to the composition of the vegetable fats in coconut and linseed oils.[34] The ordinary brown laundry soap when used in a strong suds, 0.5 per cent solution, will destroy pneumonococci and streptococci, so that dishes or clothes washed in such a water are rendered germ free.[36]

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CHAPTER XXXIII

BACTERIA IN THE SOIL

The Numbers of Bacteria in the Soil.—As soon as the plate method was devised for determining the number of organisms, it was used for counting the number of bacteria in soil. Miquel[48] determined the numbers in soil and established a gram as the amount of soil to be used as the unit, although Pasteur and Joubert[53] had already noted that the lower layers of the soil were practically sterile as compared to the upper.

Obtaining the Numbers in a Gram of Soil.—The usual method is to suspend a gram of the soil in a flask of sterile water, the suspended particles being made as small as possible, and the dilution equalized, by repeated shaking. Any ordinary shaking method will probably never give the total number present in soil, since it is impossible to separate the organisms from the gel surrounding the soil particles and in which they are imbedded. All counts that are made are probably only approximate and comparative. Whittles,[73] agitating the 1 to 100,000,000 dilution of a soil by means of a shaker attached to an electric clock, claimed that his counts were more nearly correct. Smith and Worden,[61] not obtaining concordant results from plate counts made from such a suspension with hand shaking, attributed Whittles' very high counts to imperfect sterilization of the vibrator.

No branch of microbiology better illustrates the dependence of results on methods than do soil studies, and a large percentage of the papers are only a comparison of methods and their accuracy.

Conditions Necessary for Bacterial Life in the Soil.—Soil bacteria must obtain *moisture*, *food*, and *air*, and the only position where they can get all these three requisites is in the upper foot of soil. Conditions are apparently best at a depth of about 4 to 6 inches, where the danger of drying out is reduced to a minimum, and where air from the surface can penetrate. The food is distributed in the upper layers in most types of soil, so that this limiting factor also confines the organisms to the first foot.

Numbers of Bacteria at Various Depths.—The number of bacteria diminishes in the depths of soil below 9 to 12 inches, so that, at 10 feet, organisms are scarce.[55,19,20] High counts are confined to the upper 4 to 6 inches of the soil and the numbers decline from 1,630,000 at that depth to 4,000 at 24 inches. The numbers seem to vary with the cultivation of the soil, stirring the soil increasing the numbers to be found.

Typical Iowa soil[9] shows the same decrease in the numbers of bacteria in the lower layer of the soil, indicating that although the *food and water* are abundant to some depth (3 feet), the number of organisms diminish rapidly below the top layer (Fig. 149). The other factor, *air*, would seem here to play the leading rôle. It is to

be noted of course, that all plate counts reckon only ærobic types of organisms which will grow in the ordinary culture media. This type, however, is undoubtedly the predominant one since the chief changes which are going on in the upper layers of the soil have to do with the destruction of protein and carbon combinations which have been left on or in the soil by the last crop. In soil where the food is abundant to quite a depth, as on land on which sewage has been, or is being, poured, the high

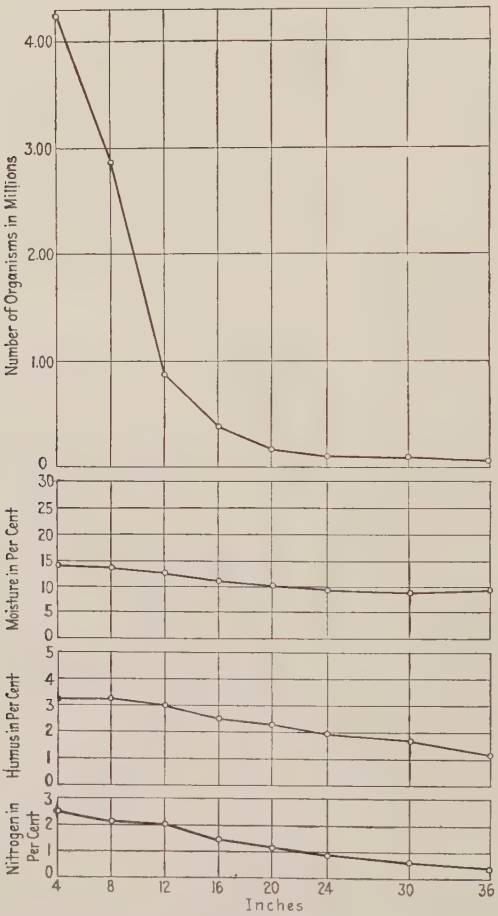

Fig. 149.—Number of bacteria in soil at various depths correlated with moisture, humus, and nitrogen. (*From Brown.*)

counts may continue to a depth of several meters or more, 45,000,000 at 10 feet; 10,000,000 at 12 feet; and 8,000,00 at 25 feet. The number of organisms in the upper layers usually lies between 1,000,000 and 10,000,000 per gram, although this number varies with the type of soil and the tillage.

Cultivation Increases the Number of Bacteria.—Cultivated soils may have twice as many bacteria as similar virgin soils, also many more are usually found in soil which has been recently tilled and is mellow,

such as wheat land as compared to alfalfa pastures which have not been recently disturbed.[24]

Liming helps in this destruction and the numbers of bacteria increase in limed soils,[9] since lime tends to neutralize the effects on the organisms of acid compounds formed where humus destruction is slow and the soil, acid. The advisability of hastening this destruction of the organic compounds in the soil is one which must be settled by the practical agriculturalist, but a permanent supply of humus (the partially destroyed organic remains of plants) is necessary in many soils for the retention of moisture.

Acidity and Alkalinity in Soil.—The neutralization of an acid condition by the addition of lime is a very simple process, but the reverse, *i.e.* the production of acidity, while advisable at times in soils, is more difficult, and no better method has been suggested than the addition of sulphur which, by oxidation and the formation of sulphuric acid, brings about the desired change, or the continued application of ammonium sulphate as a source of nitrogen.

The oxidation of sulphur in the soil is due to bacilli[71,72] with the formation of sulphuric acid.[63] The energy for the assimilation must be derived from the sulphur and sodium thiosulphate compounds destroyed, the liberated energy allowing a small amount for the assimilation of carbon supplied from carbon dioxide in the soil. The sulphur organisms are very important in the production of the acid condition in the soil from the oxidation of sulphur.

The sulphur is oxidized to form sulfuric acid:

$$2S + 3O_2 + 2H_2O = 2H_2SO_4$$

The thiosulfate is oxidized possibly to free sulphur ($Na_2S_2O_3 + O = Na_2SO_4 + S$) before the preceding reaction is completed and sulphuric acid formed.

Our lack of ready control of acidity in soil is unfortunate, as some crops are favored by a neutral or acid reaction, and a change in the soil, rendering it acid, might prevent root or tuber infections, such as potato scab.

The Numbers of Bacteria in Soil at Various Seasons.—Conn[15] has made the observation, as the result of plating soil from the same plot for an entire year, that the number of bacteria increases during the winter months (Fig. 150), and is confident that there is an actual growth of the organisms due to their activity during this season. The increase in numbers seems to be largely independent of moisture content. While the bacteria appear to increase in numbers during the winter months, this increase may be more *apparent* than *real* and due to the breaking up of clumps of bacteria in the frozen soil.[70] These broken up clumps give higher counts on agar plates; a large clump produces only one colony on a plate, broken up it might produce a hundred. The water frozen in the soil would have a tendency to expand as it thawed and to release the organisms from the clumps in which they were entangled. Two types of

flora in the soil, winter and summer, have been suggested[10] and the ammonification is more rapid and nitrification weaker during the winter months. There would seem to be, however, no difference between summer and winter floras, and bacterial activities are limited by the physical condition of the water in the soil.[39,40]

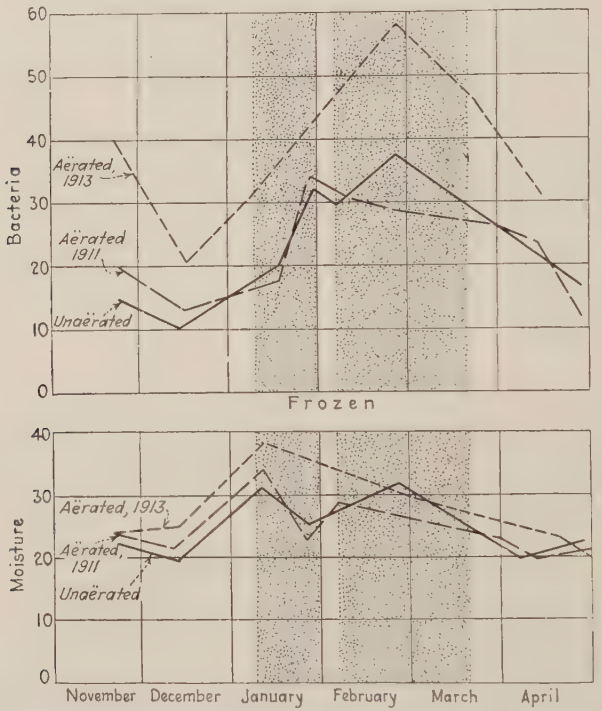

FIG. 150.—Bacteria and moisture in soil during winter months. The period during which ground is frozen indicated by shading. (*Redrawn from Conn.*)

The processes which occur in sewage are very similar to those in soil; the effluents which came from the sand filter[13] contain large numbers of ammonifying and denitrifying bacteria during the winter season, so that the sewage is imperfectly nitrified as it passes through the sand bed. The ammonifying and denitrifying process may be the predominant one in soil during the winter, with the nitrifying organisms renewing activity, as the ground warms up in the summer. The experimental basis for such a suggestion, however, is very slight.

Cell Enzymes in the Winter.—*The extracellular enzymes* secreted by the bacteria continue in the soil during the winter and, since enzymes are not so susceptible to cold, may produce some changes, although bacterial activity is at a standstill.[67] The enzymes held inside the bacteria may not function during the winter; the bacteria do not grow or increase in numbers; but enzymes already in the soil might continue to act.

The Soil Protozoa as Enemies of the Bacteria.—The possibility exists, also, that the apparent numerical increase of bacteria might be due to the absence of the natural enemies of the bacteria during the winter season. Many of the soil organisms are

eaten by the soil protozoans,[57] but such organisms could not function during the winter months, since they require warm temperatures for their active growth. After partial sterilization, soil becomes apparently more fertile and has much more available plant food than untreated soil.[58] One explanation offered is that the soil protozoa, which in normal soil play an important rôle in ingesting bacteria, are killed off by partial sterilization, and the soil organisms, having a clear field, grow rapidly and produce the soil changes in an unusually short time.

This theory has been investigated by adding various protozoa to soil itself or to soil extract with little or no effect; ammonification, nitrification, and free nitrogen fixation are not changed.[59,27] The soil protozoa undoubtedly play a part in the destruction of soil organisms, but probably only under special conditions of moisture and temperature.[60]

The moisture content of the soil has much to do with the number of protozoa present in the soil. The soil must become soaked before the cysts burst and the organisms come out as free living forms capable of destroying bacteria. Under ordinary field conditions, this amount of water in the soil is only rarely found and, therefore, the protozoa will play a very minor part in bacterial changes, although they may be present in numbers in an encysted form.[36]

Partial Sterilization and Disinfection Increase the Numbers.—The rapid increase in bacterial numbers which appear after soil has been partially sterilized may be the result of the release of new food supplies. Matthews[45] suggested that the volatile antiseptics could be utilized in this manner and Gray and Thornton[23] have described bacteria which use as food the material released from the antiseptics.

Counting Organisms Which Will Not Grow on Ordinary Media.—The ordinary nutrient agar allows only certain types of bacteria to develop. Winogradsky had already found that the nitrogen-fixing types, which grow only in the absence of nitrogenous substances, fail to develop in media rich in peptone. Moreover, the water relations and the bacterial relations to the soil particles cannot be duplicated exactly by cultural methods, even in liquids from the soil itself. In order to check the counts made by the plate method, two other procedures have been suggested:

1. *The high-dilution method*, similar to that employed by Lister for obtaining pure cultures of the sour-milk organism, first suggested for soil by Hiltner and Störmer[24] and extensively used by Löhnis and Westermann.[42] The organisms are grown in tubes or flasks of liquid media. The results do not vary much from those obtained with plates although, in general, somewhat higher.[41]

2. *The direct-count method* proposed by Conn.[16] By this method a little gelatin, 0.15 gram in 1,000 cubic centimeters of water, is used as a fixative for the soil dilution. The gram of soil is diluted with this solution in varying proportions, depending on the types of soil, from 1 to 3 or 1 to 5 for sandy soils, to 1 to 10 for clay. The soil and the solution are thoroughly mixed by shaking, and a smear made with $\frac{1}{10}$ cubic centimeter of it over 1 square centimeter on a glass slide. The smear is dried as quickly as possible over a steam bath and then stained with rose Bengal in carbolic acid (1 gram in 100 cubic centimeters) for 1 minute, part of the time over the steam bath. A brief immersion in water and air drying make the preparation ready for examination under the oil immersion lens. The bacteria are stained pink or red while the soil particles remain brown or colorless. Conn made counts of the organisms in soil of various types, by this procedure, and compared them with agar and gelatin plate counts, with the following results:

Date, 1917	Soil	Plate count		Microscopic count	
		Gelatin agar		Groups	Individuals
		Colonies	Colonies		
May 15 .	Cultivated clay loam	17,500,000	23,000,000	140,000,000	175,000,000
Aug. 10..	Cultivated silt loam	3,800,000	8,500,000	97,000,000	144,000,000
Nov. 14..	Cultivated sandy loam	13,500,000	18,000,000	248,000,000	390,000,000
Nov. 21..	Cultivated silty loam	11,000,000	24,000,000	260,000,000	405,000,000
Nov. 23..	Cultivated silt loam	5,800,000	5,000,000	174,000,000	230,000,000

The group counts included each clump as well as each isolated individual and, if all were living organisms, should correspond to the counts obtained on the plates. The average in the group was 1.5 organisms. The numbers which appear on plates, whether gelatin or agar, represented only about 2 to 12 per cent of the total numbers which would be seen with the microscope, so it must be assumed that plating methods give a rather vague notion of the numbers present in soil.

A classification of the bacteria according to shape, made at the same time, is given in the following table:

Date	Soil	Bacteria per gram, short rods	Determined microscopically	
			Medium-sized rods	Spores
May 15.....	Cultivated clay loam	167,000,000	3,000,000	5,000,000
Aug. 10.....	Cultivated silt loam	140,000,000	3,500,000	1,000,000
Nov. 14.....	Sod, fine sandy loam	375,000,000	12,000,000	3,000,000
Nov. 21.....	Cultivated silty clay loam	390,000,000	7,000,000	6,000,000
Nov. 23.....	Cultivated silt loam	228,000,000	1,500,000	750,000

The above figures are interesting to the student of the bacterial life cycles since they show that the predominant forms to be found under natural conditions are rods of various lengths, together with their spores, all being very similar to those obtained on ordinary culture media. Conn was unable to find any evidence of mold mycelium in the soil, although the molds can always be isolated on plates from any sample. As he points out, however, the number present in such a small sample would probably not be sufficient to allow one spore, or piece of mycelium, to each field examined. The Actinomycetes occur on these stained smears both as conidia and as filaments, the former much more numerous than the latter, as would be expected, but with occasional fragments of mycelium. On soil plates, however, each conidium would produce a colony, just as well as a filament, and would give the impression that the soil was full of active working Actinomycetes, while, as a matter of fact conidia probably represent a resting stage in the Actinomycete. Any plate counts on Actinomycetes in the soil are accordingly very deceptive and inaccurate.

The Life of the Bacteria in the Soil.—Conn's work is very important as it throws some light on the life of the bacteria in the soil. Many, if not all, of the bacteria pass part of their existence in the soil. They may become parasitic at times but, if food is lacking or they find their way into dust or the soil, they may survive there for some

time, either in a quiescent state or as common saprophytes. Conn has given us a glimpse of this phase of their existence and it is to be hoped that the application of this method will enable us to see the bacteria in action in the soil,[65,76,77] especially as it may be still further improved.

Bacteria, although they may not be in the spore form, will remain alive in air-dry soil for a very long time, especially in rich clay loam,[21] probably owing to the fact that soil particles retain some hygroscopic water, from which it is very difficult to free them. In fact each soil particle is surrounded, unless it has been heated, with a very thin film of water, and since the soil particles are very small and closely applied to each other, there is no reason for doubting that organisms may not find sufficient moisture between them to survive. The organic matter always present in loam soil would also have a protective influence, as would the soluble substances which come from the humus.[7]

Some organisms are more resistant to drying than others and the equilibrium between species which has been established when the soil had a high-moisture content may be disturbed and a very modified flora come to be predominant after a drought.[37]

The Physiological Culturing of the Soil Bacteria.—Since soil plates give only the number of bacteria, regardless of their function, and make no account of an important group of organisms, the nitrite and nitrate bacteria, other means have been devised to determine the numbers in a sample of soil by the amount of work that they were able to do in a certain time at a certain temperature.

Remy[56] used from 1 to 10 grams of the soil to be tested as an inoculum in a sterile solution of known composition. In 1 per cent peptone solution inoculated with 1 gram of soil, the amount of ammonia could be determined for a series of days to ascertain the rapidity with which the organisms in this particular sample were able to break up the peptone into ammonium compounds. Or, a solution of ammonium compounds could be substituted and inoculated with 1 gram of the soil. The rate at which the nitrite formers could convert the ammonia into nitrites, measured by the nitrite test, could be obtained every week or 10 days. In the same way, nitrite compounds could be used for conversion into nitrates.

Hiltner and Störmer[28] used similar solutions but added a smaller sample of the soil as an inoculum, the amount varying between 1 gram and a thousandth of 1 gram, with the tendency toward the smaller amounts. With this small amount of inoculum, there is always danger that the total number of organisms will be so small that some varieties will be missing and that all the reactions will not occur. If the organisms are so few in number, however, that they do not produce the reactions in cultures, they would not do so to any extent in the soil.

These two methods have yielded important results in spite of their obvious crudities and much has been learned about the work of bacteria in soils with various physical and chemical properties.

Cultures solutions used in flasks do not reproduce soil conditions since they do not furnish the same solid substratum of soil particles with the soil solution held between them.

The conditions in a soil extract flask or in a flask where the chemicals were held in solution with the soil added would be similar to those found in a water-logged soil where the aeration was entirely lacking. Coleman[14] and Stevens and Withers[64] have tried to reproduce natural conditions by applying various solutions to samples of previously sterilized soil. This method introduces *a second factor; the soil used as a basis for the solutions employed,* but Stevens and Withers were satisfied that this was more than offset by the advantage of a physiologically similar medium. Many soils which easily ammonify ammonium sulphate or cottonseed meal would not do so if they were added to soil flasks containing too much water, and nitrification was very slight in soils saturated with water.

THE TYPES OF BACTERIA IN THE SOIL

The earlier work on soil bacteriology was devoted largely to determining the number of bacteria which could be obtained in different types of soil, and in soil at different depths, but it soon came to be recognized that, while such counts had some value, they gave no clue to the actual work going on in the soil. The soil bacteria are now classified physiologically and described in terms of the changes which they induce. They may be divided into the following groups:

1. *The ammonifying organisms* which break up the proteins in the plants and animals which find their way into the soil.
2. *The nitrifying organisms* which convert the ammonium compounds into (*a*) nitrites, and (*b*) nitrates.
3. *The denitrifying organisms* which destroy nitrates and produce ammonia.
4. *The urea-destroying organisms.*
5. *The cellulose-destroying types* which break up the cellulose of plant tissues and release the carbon contained in them.
6. *The free-living nitrogen-fixing organisms* derive some energy from the destruction of cellulose, sugars, or other carbon compounds in the soil, and combine part of them with the nitrogen of the air to form their body proteins. A number of different species occur, most of which are unrelated.
7. *The symbiotic organisms live in the roots of the leguminous plants* from which they derive their carbohydrates. They combine carbohydrates of the plant with the nitrogen of the air to form their proteins. Part of this combined nitrogen finds its way into the tissues of the legume in which they are living.
8. *The pin-point colony formers;* very numerous but of which very little is known.
9. The *anærobic* species.

1 and 2. *The ammonifying and nitrifying organisms* have already been discussed in a previous section. These organisms are the active agents in returning to the soil the nitrogen compounds which have found a resting place in the soil in the form of the proteins of plants and animals. They add nothing *new* to the store of nitrogen in the soil, but render the complex compounds again available for the absorption by the higher plants. Not all of this nitrogen is returned to the green plants; some of it is lost by *denitrifiers.*

3. *The denitrifiers* break down the nitrates and nitrites to form ammonium compounds, many are volatile, escape into the air, and are lost to the soil. The denitrifiers include many of the common saprophytic

organisms such as *Bact. stützeri*, *B. hartlebii*, *Bact. denitrificans*, as well as *B. fluorescens*, *B. mycoides*, etc. The denitrifying function of such bacteria is a temporary one at a stage in their existence when food is scarce; they make use of the nitrates, if necessary, but do so at the expense of other plants.

4. *The urea-destroying organisms* are another type of the ammonifying species already discussed. At one time it was believed that these organisms represented a special group whose function was the destruction of the urea, but Löhnis and Fred[43] suggest that the supposed species of Miquel are only varieties of rather common soil forms everywhere present. After the urea is ammonified, the further nitrite- and nitrate-forming processes follow.

PECTOSE- AND CELLULOSE-DESTROYING MICROORGANISMS

5. *The cellulose-destroying types* have not been discussed. They form an interesting group which break up not only cellulose but related compounds. The first membrane which is formed between plant cells when they divide is composed of a carbohydrate of the composition ($C_{16}H_{24}O_{16}$), known as "pectose," which is fairly soluble in hot acid solutions. This substance lies between the real cell walls laid down later and composed of another carbohydrate, cellulose ($C_6H_{10}O_5$). The cell walls of the wood of the trees, such as the oak, beech, fir, pine, etc., are further strengthened by the impregnation of the original cellulose walls by another compound, *lignin*. About half of the dry weight of wood, such as beech, is composed of this secondary material. These very durable woody elements are not readily destroyed by the action of bacteria or other organisms.

The pectose of plants is destroyed by a series of organisms which secrete the enzyme, pectinase; among these destroyers, being the group of soft-rot organisms noted previously. *B. amylobacter*, or a member of this group known to Beijerinck and van Delden[6] as *Granulobacter pectinorum*, is also very active in its secretion of pectinase and is believed to play an important rôle in the retting of flax. The flax plants are placed under water on low land until the fibers easily can be separated from the remaining tissues, when the plants are scutched by drawing them through a toothed frame. This destruction of the material which holds the cells together is accomplished by the action of pectinase probably secreted by a number of organisms, particularly by *G. pectinovorum* under anærobic conditions. Other ærobic bacteria usually accompany this organism, and may largely help to render the conditions anærobic, and favor its growth. The pectin is broken down into simpler sugars, such as galactose, xylose, and, perhaps, also glucose and arabinose, by hydrolysis. The retting of flax and hemp is of much practical importance since it gives a simple and easy means for the separation of the fibers,

and the spreading of pure cultures of the rot organisms over the retting ground may hasten the process. Under ærobic conditions, a very different set of organisms, probably mostly molds, perform the same function.[5]

The Cellulose-destroying Types.—These are among the more important destroyers of the remains of plant life. They are of importance not only in the soil but also in the intestinal tract of the herbivorous animals, since the digestion of the cellulose by these animals is, probably, to quite an extent if not entirely, through the agency of bacteria. The cell walls of the higher plants, even of trees, are largely composed of cellulose. The walls of the parenchymatous cells which make up the bulk of the fleshy parts of plants are almost pure cellulose, but even the lignified portions have a large percentage, being almost half cellulose, so the destruction of this substance is very important.

Cellulose s not a definite chemical but composes a group of substances which give similar reactions, such as a blue coloration with zinc-chlor-iodide, or with iodine and sulphuric acid. The formula given for cellulose $(C_6H_{10}O_5)_n$, may also be written $(C_6H_{10}O_5)$, indicating that the number of times carbon, hydrogen, and oxygen are taken, although in the portion given, is not known. The cellulose may vary depending on its building up by the hydrolysis of different sugars such as glucose, mannose, or xylose.

Van Tiegham[68], [69] had observed that *B. amylobacter* would digest not only starch, as its name would indicate, but would also dissolve the cell walls of the softer portions of plants, such as the tips of the stems and roots. This bacillus, therefore, was the first discovered fermenter of cellulose. Popoff[54] confirmed the generally accepted observation that one of the most important gases formed above stagnant water or sewage, where fermentation was occurring, was marsh gas (CH_4). He did not use pure cultures of bacteria, as his experiments antedated them, but simply mixed slime from the sewers of Strassburg with water, and carefully collected the gas above mercury. That the process was a vital one was demonstrated by the addition of antiseptics to the slime when gas formation ceased. Slime inoculated into water containing Swedish filter paper (pure cellulose) and the gases analyzed gave the following results:

Gases found	After 2 weeks, per cent	After several weeks, per cent
Carbon dioxide	25.70	34.07
Methane	14.42	37.12
Hydrogen	14.36	1.06
Nitrogen	45.52	27.75

It will be noted from this table that the production of methane increases and that of hydrogen greatly decreases after the first 2 weeks of fermentation.

Hoppe-Seyler[29], the physiologist, interested himself in 1881 in the fermentation of cellulose. He placed 25.773 grams of cellulose filter paper in a 1-liter flask, inocu-

lated it with a little sewage slime, and then made it up to 700 cubic centimeters on Dec. 2, 1881. The flask was kept in the darkness during the 4 years the experiment lasted. The first 2 years the fermentation of cellulose was very active but slackened down so that, at the end of 3½ years, it was hardly apparent, and the experiment was concluded on Dec. 6, 1885. During the 4 years, as the result of 95 gas determinations, it was found that 3,281 cubic centimeters of carbon dioxide had been produced and 2,571 cubic centimeters of methane; 15 grams of the cellulose had been consumed and no by-products could be found in the solution, apparently all having passed off into the air. He suggested that the first reaction was the hydration of the cellulose.

$$C_6H_{10}O_5 + H_2O = C_6H_{12}O_6$$

The second phase was the destruction of this carbohydrate, presumably a sugar, into carbon dioxide and methane.

$$C_6H_{12}O_6 = 3CO_2 + 3CH_4$$

The two gases were produced in approximately these proportions, so that the equation seems very plausible.

Omelianski's Cellulose-fermenting Bacteria.—The special name connected with the investigation of the destruction of vegetable cellulose, however, is that of Omelianski, who published his work in two phases.[50,51,52] This Russian investigator isolated, from the slime of the River Neva, an organism which, growing anærobically in culture media containing only cellulose filter paper (Fig. 151) with a minimum of nutrient salts, would ferment cellulose with the formation of hydrogen.

FIG. 151.—Strips of filter paper dissolved in spots to form holes by cellulose-destroying bacteria. (*Omelianski.*)

The method used was to enrich his cultures by a modification of the Winogradsky technic developed for the isolation of the nitrite and nitrate organisms, the first step being to secure as nearly as possible a great predominance of cellulose-destroying bacteria. This end was secured by growing them from the river slime, where they occurred, along with other organisms, in a culture medium where they could have an optimum chance to grow, and other contaminating species would find it difficult to persist. Such a medium would have the necessary nutrient salts, a very little organic nitrogen, and an abundance of cellulose. The protein-fermenting types finding little upon which to grow, slowly disappeared in repeated transfers. After an approxi-

mately pure liquid culture of the cellulose organism had been obtained, trials were made to determine the amount of gas produced, and to compare this gas with the amount of cellulose used. Some calcium carbonate was added to the solution to maintain the alkalinity, as the organism produces citric, butyric, and valerianic acids, rendering the medium acid and stopping the reaction, after 67 per cent of the cellulose had been converted into fatty acids and 30 per cent released as gas. The gaseous products were about 98½ per cent of carbon dioxide and 1½ per cent of hydrogen by weight. By volume, there would be about three times as much carbon dioxide as hydrogen for the entire course of the fermentation, but the relative proportions of the two gases varied greatly during the early and late stages.

Omelianski's second paper[51] announced that he had been able to isolate a second organism which would ferment cellulose but with the formation of methane alone. In his previous experiments he had noted that, during the early phases of the isolation, some methane was developed, but as purification of the culture went on, this production of methane finally ceased, so he decided that it was due to another organism which later disappeared. He now found, in his later work, that if he gave the methane and cellulose organisms time to sporulate in slime used for inoculation, the methane formers would sporulate, and he could free his culture from the non-sporulating hydrogen types. A long slender organism was then found to be predominant in the culture, although other species still persisted. Morphologically, the methane and hydrogen forms were very similar but the gases produced were dissimilar. Omelianski was not working with absolutely pure cultures in the trials where he secured the hydrogen gas and the fermentation may have been the result of the association of several forms.

Both Anærobes and Ærobes Are Cellulose Fermenters.—The above experiments were performed, it will be noted, entirely on anærobes, but van Iterson[30] called attention to the fact that ærobes could also attack cellulose. In an alkaline medium, the organisms are bacteria; in an acid medium, they are molds and higher fungi. More recent work on cellulose-destroying ærobes is contained in a series of papers by Kellerman and McBeth;[34] McBeth and Scales;[46] and Kellerman, McBeth, Scales, and Smith.[35] In the first paper, the authors investigated the original cultures of Omelianski which are still in existence and found that they were far from pure, containing a variety of organisms, among which they isolated three new species of cellulose-fermenting bacteria.

These investigators made one important advance in the methods for the study of cellulose organisms by the use of cellulose agar for obtaining pure cultures. The preliminary enrichment was similar to Omelianski's, but after a fairly pure liquid culture had been obtained, it was plated out on agar in which was suspended a quantity of finely precipitated cellulose with the necessary inorganic salts. This cellulose agar, was opaque when in the Petri dishes but, as cellulose-dissolving colonies grew on it, the region surrounding them became transparent. This fact was no guarantee, however, that the culture was a pure one, as many accompanying organisms, unable to ferment cellulose, lived on the cellulose products of the fermenting species. On this agar they isolated two new species of cellulose destroyers from Omelianski's culture of hydrogen organisms and one new one from his methane culture in addition to a number of contaminating organisms. All these isolated organisms were ærobic, although Omelianski was of the opinion that his organisms were purely anærobic in their action.

McBeth and Scales[46] and Kellerman, McBeth, Scales, and Smith,[35] using this agar and also starch agar (the cellulose-destroying forms also grow on starch agar), isolated over 20 species of cellulose fermenters from soils from various parts of the United States. These species, they claim, they were able to distinguish from each other by their size, number of flagella, and physiological features.

Of 50 soils examined from different sections of this country, all showed from one to three ærobic cellulose destroyers present. None of them formed gases from the reaction so that they represented an entirely different group from that with which Omelianski had previously worked, since his organisms were anærobic and formed either methane or hydrogen.

An unusual species of this sort is the *Microspira agar-liquefaciens*, isolated by Gray and Chalmers,[22] an ærobe which will digest not only cellulose, lignin, and xylose, but which will liquefy agar, a very rare occurrence among bacteria.

A more recent formula for the growth of cellulose-destroying bacteria is slightly alkaline (pH 7.5) and contains: $NaNO_3$, 0.5 gram; K_2HPO_4, 1 gram; $MgSO_47H_2O$, 0.5 gram; KCl, 0.5 gram; $FeSO_4 \cdot 7H_2O$, 0.01 gram; distilled water, 1,000 grams. Five cubic centimeters of this medium is introduced into test tubes containing a partially immersed slip of filter paper.[18]

The Molds as Cellulose Fermenters.—The ordinary molds also play an important part in the destruction of the cellulose in the soil, being especially active near the surface of light, well-ærated soils.[8] Many common species are very active cellulose destroyers according to McBeth and Scales[46] who tried out the various species of Penicillium which Thom[66] had accumulated in his investigations of that group. Six of the species were cellulose destroyers. In addition to the Penicilliums, a number of other common forms, such as *Fusarium* and *Cephalothecium roseum*, were also active. In light, well-ærated soils, there can be no question but that they are the most active agents in cellulose destruction; in heavy soils, except at the surface, the bacteria play the leading rôle.

FREE-LIVING NITROGEN FIXERS

6. Nitrogen-fixation and Soil Carbohydrates.—The removal of nitrates from the soil by repeated cropping with wheat or rye takes away nitrogen and this loss, together with the constant leaching of soil nitrates by soil water, would render, in the course of a period of years, many soils infertile from a lack of nitrogen compounds. In fallow soils, the nitrogen content can be maintained by the *free-living bacteria*, but they are not able to do this in cropped soil. These organisms are called *free-living*, because they are not dependent on other plants for part of their food. They combine the nitrogen of the air with some soil carbohydrates to form proteins. This synthetic process consumes much energy for the nitrogen fixed, so soil in which these organisms grow must be supplied with carbohydrates in a readily available form. Since sugars of any type are very readily attacked by molds and bacteria, McBeth[47] suggests cellulose as the carbohydrate utilized by the nitrogen-fixing bacteria.

Cellulose-destroying bacteria require an abundant supply of nitrogen so that the two sets of organisms may be regarded as working more or less in symbiosis, the free-living nitrogen fixers supplying nitrates, and the cellulose destroyers adding soluble carbohydrates. Winogradsky[74,75] found that, from 3 grams of dextrose, he secured the fixation of 2.1 to 21.2 milligrams of nitrogen in the form of ammonium sulphate when he used the anærobic, butyric-acid bacterium as the free-fixing organism. He estimates, therefore, that the ratio of nitrogen to carbohydrate would not be greater than 6 to 1,000. Winogradsky did not use pure cultures, however, and among the organisms composing his mixed cultures were, undoubtedly, many denitrifying types

whose activities reduced the apparent total amount of fixed nitrogen, converting it into ammonia which passed off into the air. The amount of energy used may not, however, be as large as Winogradsky believed.[11]

The Free-living Organisms Producing Fixed Nitrogen.—The organisms which are able to fix nitrogen from the air may be divided into two classes, the anærobes and ærobes. The anærobes are of the type of the butyric-acid organism. Other organisms may also be able to fix nitrogen, but all investigational work seems to have been among the *B. amylobacter* group.

The ærobes are more numerous, including not only ærobic bacteria but also molds, although there seems to be much doubt as to the importance of the latter. The discovery of *Azotobacter chroococcum* and *Azotobacter agilis* by Beijerinck[4], ærobic free-living species, and of three other species, *A. vinelandii*, *A. beijerincki*, and *A. woodstownii* by Lipman[38] in New Jersey soils, and of *A. vitreus* by Löhnis and Westermann[42] revealed an entirely new group of organisms which, if properly fed with carbohydrates, could fix atmospheric nitrogen; 54 per cent of the soils examined by Niemeyer[49] contained Azotobacter. These organisms are much more efficient in their utilization of carbohydrates, fixing two to three times as much nitrogen for a given amount of carbohydrate consumed as do the anærobes. They are short rods, of the shape of a coffee bean, motile by means of 4 to 10 polar flagella in *A. agilis;* by a single polar flagellum in the others, except *A. vitreus* which is non-motile (Figs. 152, 153).

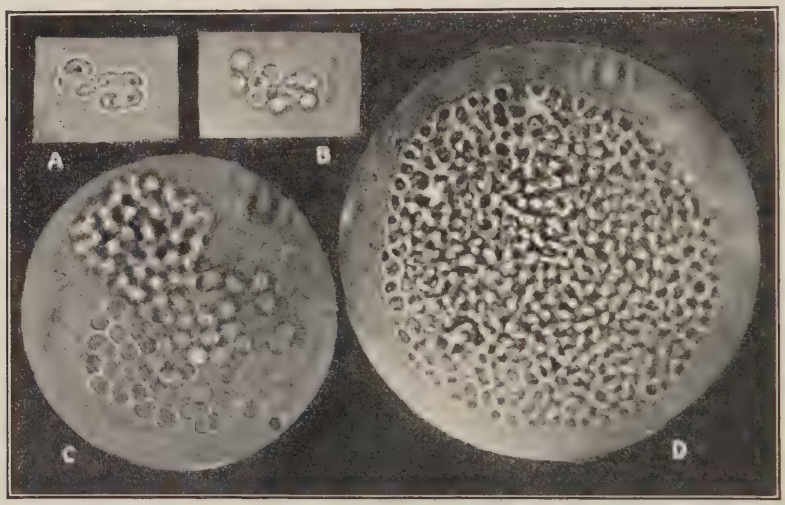

FIG. 152.—Colony growth in Azotobacter: *A*, after 16 hours; *B*, 36 hours; *C*, 60 hours; *D*, 4 days. × 1,000. (*Photographs by Prof. D. H. Jones.*)

Beijerinck[4] isolated them from enrichment cultures consisting of 100 cubic centimeters of tap water, 2 grams of mannite, and 0.02 gram of K_2HPO_4 with a little garden soil. After 2 or 3 days, a scum appeared on the surface of the liquid which continued for a long time and contained short bacilli with rounded ends. They were plated on an agar made up of essentially the same substances as the liquid enrichment cultures. Other sugars, such as saccharose, glucose, maltose, or galactose, could be substituted as a source of energy for mannite, but glycerine would not permit rapid growth.

The interesting suggestion has been made that while nitrogen may be fixed from the air as nitrates, or gathered from the soil in the same form, the nitrates and sugar may form an intermediate product, a sugar nitrate, the chief function of the Azotobacter in the soil being to save the nitrate-sugar compound from denitrification by combining it into a more stable chemical.[3]

The Organisms.—The organisms are large rods, or at times, almost spheres, the appearance depending on the culture. A series of cultures have to be examined to determine the form and species.

Jones[31,32,33] first described some of the changes that the Azotobacters undergo internally in old cultures (Fig. 153). Granules are present after 3 to 4 days, some apparently glycogen; but others of chromatin staining with anilin dyes. The latter granules seem to be derived from a small body to be seen in each young cell. They divide just before cell fission. The secondary granules escape from the old cells and seem to give rise to new individuals. Part of these organisms are enclosed in capsules and as fission often occurs inside these capsules, a group of from two to six organisms is formed. These bodies are not true endospores, as they lack a thickened wall, but they serve to carry the organism over periods of drought, and grow into normal cells when water is again supplied.[2]

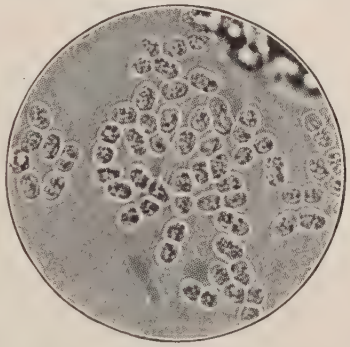

FIG. 153.—Free-swimming cells of Azotobacter, breaking away from the parent colony. × 1,000. (*Photograph by Prof. D. H. Jones.*)

The interesting and suggestive work on life cycles in these organisms by Löhnis and Smith[44] will be discussed later in a special chapter.

Azotobacter grows slowly in culture media as is shown by a series of photographs, taken by Jones[31] (Fig. 152), of colonies 16 to 96 hours old.

While *Azotobacter chroococcum* has often been grown in pure cultures, the study of its reactions on solid media is very difficult, as agar seems to introduce a colloidal factor which prevents normal development; phosphates are absolutely indispensable and the organism will not grow if the medium becomes acid.[1]

Azotobacter Alone and in Combination with Other Bacteria.—Beijerinck and van Delden[6] grew *A. chroococcum* in symbiotic association with an organism often found with it in the liquid cultures previously described. This organism was named *B. radiobacter* as it formed star-shaped colonies of long slender rods. *A. chroococcum* and *B. radiobacter*, when grown together, fixed over fifteen times the amount of nitrogen that *A. chroococcum* did alone. The highest yield of fixed nitrogen, however, was from a combination of three organisms: *A. chroococcum*, *B. granulobacter*, and *B. radiobacter*. The process here is undoubtedly a symbiotic one since the *B. granulobacter* is an anærobic nitrogen fixer, while the others are ærobes.

Work done by McBeth[47] would seem to support the theory of symbiosis, the amount of nitrogen fixed by Azotobacter being increased if it was associated with a cellulose-destroying organism, *B. rossica*, the latter bacillus supplying energy from its destruction of cellulose.

The introduction of a reducing organism as a member of the nitrogen-fixing combination would have a tendency to lessen the amount of nitrogen found in the medium at the end of the experiment, as much of the material would be converted into ammonia and pass off into the air from the activities of the reducer. In the soil, of course, the combination of organisms varies, depending to a large extent on the physical and chemical conditions in the soil at any time. If conditions favor the denitrifying group, the sugars formed in the soil from the decomposition of the cellulose are utilized for the destruction of the nitrates, since denitrifiers use carbon compounds in order to get the energy to denitrify soil,[12] and the relation between the amount of sugar and the amount of denitrification is a fairly constant one in these organisms. For example, soil should contain 1 per cent dextrose to 1.6 per cent potassium nitrate to provide the necessary sugar for the maximum reduction of the nitrate by *B. pyocyaneus* and *B. fluorescens*. If the sugar content rises above 2 per cent, the rate of denitrification falls. The absence, or almost total absence, of air is necessary to secure a high rate of denitrification, as shown by growing cultures in an atmosphere of nitrogen or of hydrogen. *Three things are necessary, therefore, for denitrification: exclusion of the air, presence of a source of energy such as sugar or cellulose, and a supply of nitrates.* These conditions occur in a heavy water-soaked soil, where there is an abundance of humus and an accumulation of nitrogen from decaying organic matter.

The best condition for nitrification, on the other hand, is a light, well-aerated soil with some humus as a source of energy.[62]

7. *The symbiotic nitrogen-fixing species* found on leguminous plants have already been discussed.

8. The Pin-point Colony Formers.—Numerous slow-growing organisms form so-called "pin-point colonies" on agar plates made from soil. The rôle which they play in the soil has not been determined, but the large numbers which are present in any sample, especially frozen soil, indicate that they are an important group.

Conn[17] has divided them into rod-shaped and coccus types but has not attempted to classify them. The possibility that organisms which appear as rods under culture conditions may in the soil be spheres has been suggested by the studies of Winogradsky.[76]

9. The Anærobic Species.—The anærobic species are numerous in the soil and act not only on the cellulose compounds but also on proteins, fix nitrogen, form nitrates, and reduce them.[25,26]

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CHAPTER XXXIV

BACTERIA IN AIR, WATER, AND ICE

BACTERIA IN AIR

One of the earliest contributions on the bacteria in the air was made by Pasteur in 1860, during the discussion as to the spontaneous origin of life, while he was trying to convince the Paris Academy of Science that bacteria did not arise from inorganic matter without preexisting life.

Pasteur, during his summer vacation in 1860, revisited his old home at Arbois and spent the time in the study of the organisms in the air at different altitudes. Its nearness to the higher Alps made comparisons between low, inhabited regions with high, uninhabited ones easy[37] (pp. 97 and 98):

"Pasteur started for Arbois, taking with him seventy-three flasks; he opened twenty of them not very far from his father's tannery, on the road to Dôle, along an old road, now a path which leads to the mount of the Bergère. The vine laborers who passed him wondered what this holiday tourist could be doing with all those phials; no one suspected that he was penetrating one of nature's greatest secrets. 'What would you have?' merrily said his old friend, Jules Vercel. 'It amuses him!' Of those twenty vessels, opened some distance away from any dwelling, eight yielded organized bodies.

"Pasteur went on to Salins and climbed Mount Poupet, 850 meters above the sea-level. Out of twenty vessels opened, only five were altered. Pasteur would have liked to have chartered a balloon in order to prove that the higher you go the fewer germs you find, and that certain zones, absolutely pure, contain none at all. It was easier to go on into the Alps.

"He arrived at Chamonix on Sept. 20, and engaged a guide to make the ascent of the Montanvert. The very next day this novel sort of expedition started. A mule carried the case of thirty-three vessels, followed very closely by Pasteur, who watched over the precious burden and walked alongside precipices, supporting the case with one hand so that it should not be shaken.

"The next morning, twenty flasks which have remained celebrated in the world of scientific investigators, were brought to the Mer de Glace. Pasteur gathered the air with infinite precautions; he used to enjoy relating these details to those people who call everything easy. After tracing with a steel point a line on the glass, careful lest dusts should become a cause of error, he began by heating the neck and fine point of the bulb in the flame of the little spirit lamp. Then raising the vessel above his head, he broke the point with steel nippers, the long end of which had also been heated in order to burn the dusts which might have been on their surfaces and which would have been driven into the vessel by the quick rush of the air. Of those twenty flasks, closed again immediately, only one was altered. 'If all the results are compared that I have obtained until now,' he wrote, on March 5, 1880, when relating this journey to the Académie, 'it seems to me that it can be affirmed that the dusts suspended in atmospheric air are the exclusive origin, the necessary condition of life in infusions.'"

The altitude and the proximity of human habitations, as will be seen from the above experiments, have much to do with the numbers of bacteria in the air.

The following figures[4] are of interest as showing how a few hundred feet in elevation greatly diminish the numbers: "At the top of the clock tower (of the House of Commons) there were only 1.3 bacteria and molds per liter, while halfway up this tower the number was 1.5. Twenty feet from the ground the number rose to 3.3 per liter, and on the ground itself 4.2 per liter were found. The same results have been obtained in other investigations of this nature. Thus, on the spire of Norwich Cathedral (310 feet), 10 liters of air yielded 7 microorganisms. Whereas, on the tower (180 feet), 9 were obtained, and on the ground 18 for the same quantity of air." The microorganisms are found as high as the dust, since they are carried by the dust particles and are, therefore, dependent upon currents of air and the velocity of those currents to waft them into the upper layers of atmosphere.

Some of the most interesting and complete observations on bacteria in the air were made by Miquel,[23] during the years 1880 to 1884 at the meteoriological observatory at Montsouris, at a time when bacteriological methods were not yet standardized. The bacteria increase twenty-fold at eight o'clock in the evening, as compared to the numbers at noon. At eight o'clock in the morning, the numbers are again high, the minimum being obtained about noon. At night the number of bacteria fall during

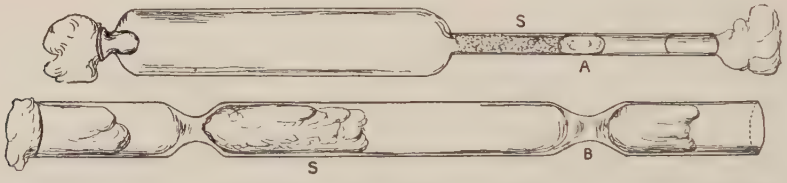

FIG. 154.—Tubes for determining the number of bacteria in air: *A*, Sedgwick and Tucker's tube; *B*, Frankland's tube. At *S* in each tube some granulated sugar. If air passes through the tube the dust and bacteria are caught by the sugar. The sugar can then be dissolved out by water and the bacteria determined by plating. (*After Swithinbank and Newman.*)

the early evening to rise again as morning comes on. The two maxima, eight o'clock in the morning and eight o'clock in the evening, are contrary to popular impressions; early morning and evening air are not the purest but the worst, so far as bacteria are concerned. Miquel ascribes these morning and evening maxima to oblique currents of air, resulting from the unequal cooling and warming of earth and air. These currents, rising, carry up the organisms from the surface of the ground.

The enormous amount of labor required for such experiments is shown by these facts: 600 to 800 preparations of beef broth every 24 hours and the services of five persons, so that there would be no interruption in the observations, were necessary. It is obvious that a mechanical device might perform the same work with much less labor, so Miquel devised an apparatus for recording the number of organisms in the air at all times of the day and night. The essential feature of this apparatus for recording was a large drum which revolved by clockwork and to which was attached a strip of paper coated with a nutrient jelly. A current of air was directed against this paper in the form of a jet, the paper being kept slightly moist so that the organisms would adhere to it. The paper had to be sterile before exposure, but after it had revolved on the drum for 24 hours it was incubated and the number of organisms counted as colonies.

Obtaining the Numbers in a Given Amount of Air.—The exposure of Petri dishes filled with nutrient agar gives an idea of the relative numbers flying in air, but does not determine the number present in a cubic meter (the standard quantity). The best apparatus is probably that of Sedgwick and Tucker,[32] a glass tube 30 to 35 centimeters in length and about 4 centimeters in width, with a neck at one end and a tapering tube at the other in which is placed some granulated sugar, No. 50, the sugar being prevented from sifting out of the tube by a very fine-meshed copper wire net near

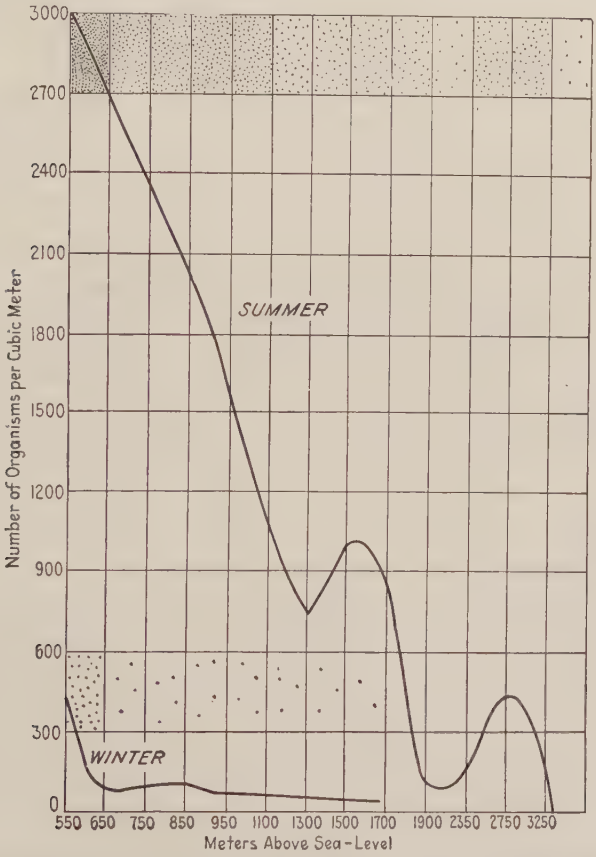

FIG. 155.—The number of bacteria in the upper layer of air as determined by 7 balloon ascensions, both summer and winter. (*After Hahn.*)

the end. Both ends of the outfit are plugged with cotton and the whole baked for 2 or 3 hours at 120°C.; a higher temperature will char the sugar (Fig. 154). In testing air, the lower part of the tube is attached to an aspirator and a definite quantity of air drawn through the tube and the sugar. Agar or gelatin may then be poured in a warm and liquid condition into the upper portion of the tube, the sugar with its enclosed germs dissolved in the media which may then be spread out in a sterile Petri dish. Or, the sugar may be dissolved out in a few cubic centimeters of warm water, added to melted agar, and poured into a plate. Washing must be repeated several times to insure the removal of all organisms.

The Organisms Are Attached to Various Sizes of Dust Particles.—A large percentage of these organisms in the dust of the air is attached to dirt particles, as pointed out by Tyndall.[36] The size of the particles determines the length of time that they will float in the air and the strength of the current of air necessary to raise them up after they have settled down. Particles of dust were divided by Flügge[7] into two types (1) those larger particles which required an air current moving at the rate of 1 centimeter per second to keep them in motion, and (2) the smaller ones which would remain suspended with currents moving 1 to 4 millimeters. With the heavier particles, a current of air moving with a speed of at least 5 meters per second would be necessary to stir them up after they had been deposited. If stirred up by persons walking or otherwise moving rapidly in a room, they would remain for some time in suspension.

In quiet air in which no movement occurs, the dust particles settle to the ground or floor. In a closed room, however, 5 hours are necessary for the very finest particles to reach the floor, and, in the open air, a much longer time would be required to float down from a distance of several hundred feet. Very fine volcanic ash sometimes floats half way around the globe and remains in the upper air for many months after a volcanic eruption, but the fine bacteria-laden dust from the earth's surface probably never goes so high.[8] It is doubtful whether ascertaining the numbers in air is of much value except scientifically, as numbers mean little. Probably not one in millions of those in suspension would have the slightest harmful effect, although a few of the disease-producing types, such as the tuberculosis organism, live for a long time in the dried sputum of a tubercular patient. These pathogenic types would occur only in the dust near the ground.

The upper layers of air are comparatively free from dust particles and living organisms, even over densely populated and cultivated land. Most observations on numbers of microorganisms in the upper air have been made in mountains and over snow-covered ground, but Hahn[11] made seven trips over South Germany, part of them in the winter when the ground was covered with snow, in a balloon especially equipped for meteorological and bacteriological observations (Fig. 155). The number of dust particles varied from 15,000 particles at the height of 640 meters to 1,500 particles per cubic meter at 1,200 meter. The number of microorganisms diminished in the upper strata of air, being influenced: (1) by their sedimentation from rain or snow falls; (2) by currents of air which waft organisms and dust upward from the lower layers; (3) by the destructive action of sunlight. The number of dust particles and bacteria parallelled each other at all elevations.

Above the city of Moscow, Mischustin[24] found four to five times as many organisms in the air as at similar altitudes over the surrounding country.

BACTERIA IN NATURAL WATERS

While the upper clouds are free from dust and organisms, as soon as raindrops or snowflakes fall into the lower atmosphere, they are contaminated and arrive on the earth's surface with an accumulation of the atmospheric organisms which they have gathered. Rain falling over a large city has a higher bacterial content than over open country. The first part of a rain has always more organisms to the cubic centimeter than the latter part which comes down after the impurities have been largely removed. Duclaux[3] states, on the authority of Tissandier, that the dust over Paris amounts to 23 milligrams per cubic meter but only 4 milligrams in air over the fields around Paris. Almost the same

proportions are shown in the bacterial number in waters which fall over Paris and the country, 19 per cubic centimeter in rain water collected in Paris and 4.3 per cubic centimeter outside.

The great contamination of water comes from contact with the earth. As it flows over fields, it picks up soil organisms and carries along thousands of them in every cubic centimeter, especially over newly ploughed land and in heavy rains. Streams become turbid with the particles of sediment that they carry, many of them containing bacteria. The majority of these organisms are harmless soil types, some die quickly in the water from lack of food, but many survive and are carried out of the surface water as the stream deposits its sediment.

More important than the soil bacteria carried into streams with surface water are the sewage organisms poured at the present day into every stream of any size. Sewage contains from 1,000,000 to 10,000,000 bacteria in every cubic centimeter, and, as many of them are derived from human source, the possibility always exists that included in these large numbers may be a few that are harmful to susceptible persons.

Natural waters, from springs and wells, are not so likely to have high numbers unless they are contaminated from the outside. The water which flows in the deeper underground channels is comparatively free from bacteria and water derived from a source of this type is probably the purest of all, as is shown by counts made of bacteria in artesian well water.

Deep Wells and Spring Water.—Prescott and Winslow[28] examined the water from deep wells and springs in 1903, with the following low counts: Worcester, Massachusetts, 10; Waltham, Massachusetts, 3; Newport, Rhode Island, 7; Saranac Lake, New York, 11; Ellenville, New York, 0; Hyde Park, Massachusetts, 12. The soil serves as a filter and the water which finds its way into underground channels has had many organisms removed from it. Shallow, farmyard wells are often contaminated from the surface waters with very high counts which find their way into them. Prescott found in Massachusetts the following number in such waters:

Bacteria per cubic centimeter..	0	1 to 10	10 to 20	20 to 50	50 to 100	100 to 500	500 to 1,000	1,000 to 2,000	Over 3,000
Number of wells.....	3	16	14	16	11	31	5	4	0

The Massachusetts State Board of Health[21] examined, about the same time, 37 springs and found an average of 41 bacteria per cubic centimeter.

While well water may have, in many cases, very low counts, the number of bacteria depends almost entirely on the drainage which it receives. If located near a barn or privy, sewage organisms may find their way through the soil for some distance and flow in a contaminating stream in rainy weather into the well. Even cement curbing

will not entirely prevent this, as contamination may occur below the top of the well, the only real safeguard being a cementing of the well down to the rock or to such a point that surface streams would not be likely to enter. The same precautions apply also to cisterns, only in a greater degree, since their proximity to houses or barns renders them especially liable to pollution.

The Bacteria in Sewage and Drinking Water.—The numbers and the influence of bacteria in sewage and in drinking water have been the subject of detailed bacteriological investigation. Bacteria in sewage are responsible for the destruction and simplification of all the organic materials which find their way into waste waters: proteins, carbohydrates, cellulose, and fat. The organisms which carry on this work are essentially the same as those which occur in the soil; there are no special species of sewage bacteria. The bacteria in drinking water are soil organisms and those from the domestic animals and man, among the latter, occasionally, some that are pathogenic. Not many bacteria are found exclusively in water, although a few species are found here only, but they are unimportant from the standpoint of the sanitary engineer.

The Disposal of Sewage.—The problem in the disposal of sewage is to render the materials soluble and simple, especially the solids. The simplest and cheapest method is to run it into a large stream or lake where the waters heavily dilute it and it gradually becomes oxidized. Such a method, while cheap, wastes all the valuable material which might be applied again to the land for growing of further crops, and endangers the health of those using the water from such streams or lakes for drinking purposes, as a few disease-producing bacteria may be found among the multitudes of harmless species.

The waste to mankind, when sewage is poured into streams, lakes, or the ocean, has never been more forcefully shown than in the chapter called "The Earth Impoverished by the Sea" of Victor Hugo's famous novel "Les Miserables."

"Paris casts twenty-five millions of francs annually into the sea. How so, and in what way? by day and night. For what object? nothing. By means of what organ? its intestines. What are its intestines? its sewers. Science, after groping for a long time, knows now that the most fertilizing and effective of manures is human manure. To employ the town in manuring the plain would be certain success, for if gold be dross, on the other hand our dross is gold.

"What is done with this golden dung? it is swept into the gulf. We send at great expense fleets of ships to collect at the southern pole the guano of petrels and penguins and cast into the sea the incalculable element of wealth which we have under our hand. All the human and animal manure which the world loses, if returned to the land instead of being thrown into the sea, would suffice to nourish the world. All this is a flowering field, it is green grass, it is mint, it is thyme and sage, it is game, it is cattle, it is the satisfied lowing of heavy kine at night, it is perfumed hay, it is gilded wheat, it is bread on your tables, it is warm blood in your veins, it is health, it is joy, it is life.

"For the nutrition of the plains produces the nourishment of men. Statistics have calculated that France alone pours every year into the Atlantic a sum of half a milliard (of francs). The cleverness of man is so great that he prefers to get rid of these five

hundred million in the gutter, the water poisoned; hunger issuing from the furrow and illness; the public wealth goes to the river and Europe is being ruined in this way by exhaustion. As for France, we have mentioned the figures. Now, as Paris contains one twenty-fifth of the whole French population, and the Parisian guano is the richest of all, we are beneath the truth when we estimate at twenty-five millions, the share of Paris in the half-milliard which France annually refuses. These twenty-five millions employed in assistance and enjoyment would double the splendor of Paris. In the blindness of a bad political economy, the people allow the comfort of all to be drowned and wasted in the waters. Paris and you will ruin yourself. But these surprising follies are not new: it is no youthful nonsense. The ancients acted like the moderns. 'The drains of Rome,' says Liebig, 'absorbed the entire welfare of the Roman peasant.' When the Campagna was ruined by the Roman drains, Rome exhausted Italy, and when it had placed Italy in its cloaca, it poured into it Sicily, and then Sardinia, and then Africa. The drains of Rome swallowed the world. Eternal city and unfathomable drain."

The impassioned appeal which Victor Hugo made in 1862 for saving the fertilizing elements would seem to have had an effect so far as Paris is concerned, for that city in 1868 established sewage farming. Berlin also disposes of its sewage in the same way and many middle-sized English cities, such as Leicester, Cambridge, Coventry, and Rugby, have done so for 50 years. Today, the entire sewage of Paris is poured out over irrigation fields located below the city on the Seine River. The city owns 3,450 acres of the land rented out to farmers who pay Paris and receive a stipulated amount of sewage, night and day while 8,650 acres more belong to individual farmers who receive sewage as they need it for their crops and pay accordingly. The privately held lands have quintupled in value where vegetables can be raised for the Paris market and also greatly increased where the only crops are grain or grass.

Berlin.—The sewage of Berlin is poured out over 22,000 acres of rather light sandy soil on which rye, wheat, barley, oats, corn, potatoes, beets, and carrots are raised at a small profit to the city.

The English cities are tending more and more to this method of sewage disposal. The English viewpoint has undergone at least three changes in the past with respect to the disposal of sewage and the contamination of streams or water courses. From 1842 to 1857, the object was to get the sewage away from the homes and into a river if necessary; from 1858 to 1870, the abandonment of the notion that sewage farming was unhealthful and the adoption of this method as being best and most economical; and from 1870 onwards, that any method which polluted streams was to be avoided. We, in America, are still in the 1842 stage in our development.

The American experience with sewage farming has not been successful, owing to inefficient management, high cost of labor, indifference of engineers to the economic problems involved, and our unstable political system.

The Fertilizer Value of Sewage.—The fertilizer value of sewage is rather difficult to ascertain, since sewage has always been analyzed for other constituents than those of commercial fertilizers, about the only components of importance in these analyses being the nitrogen and fats. The following table[10] will give some idea as to the amount of these constituents in the ash:

		London	Columbus	Chicago	Providence
Total nitrogen		13	14.4	18.5	17
Chlorine		24	32	44	
Suspended matters	total	87	98	155	150
	mineral	41	51	89	20
	organic and volatile	46	47	66	130
Fats			19.1	26	

An enormous amount of solid matter is carried out by the sewers of a great city; from Chicago, 155 grams per person daily or a city total of 137,000 tons a year. Factory and industrial wastes make considerable difference in the composition of sewage, depending on the type of manufacturing pouring its wash water or residues into a sewer system. The amount of solid matter in sewage also varies with the city; in America it is estimated at between 20 and 70 tons per capita per annum; in England, between 30 and 60 tons. The sewage in Europe is much more concentrated than that of America; we use about 250 gallons of water per day per capita while in England the average is only about 30, and sometimes as low as 6 gallons per capita per day.[19]

The amount of material from a city such as New York is shown in the following table:

Dry Suspended Solids in New York City Sewage

Material	Tons per 1,000 inhabitants annually	Tons entering New York harbor annually
Feces	14	77,600
Toilet paper and newspaper	8	44,300
Soap and washings	11	60,900
Street wastes	8	44,300
Miscellaneous	4	22,200
Total	45	249,300

The following table gives the principal constituents of sewage in grams per capita daily, although these constituents vary with different cities and different seasons of the year:

	Grams
Nitrogen as free ammonia	7
Albuminoid ammonia	2.5
Organic	8
Total	15
Chlorine	19
Fats	19
Dissolved matters	136.0
Suspended matters	93.0
Total solids	229.0

Many of the Bacteria are of Human Origin.—Particular attention should be called to the high percentage of fats, from the uncombined fats, forming as it does one of the most difficult problems in sewage disposal, since fats are among the most resistant substances to bacterial attack. In addition to the chemical substances in sewage, it has been estimated that there are present also 322,000,000,000 bacteria for each

person connected with the sewer, since about 27.95 per cent of the dry weight of feces is made up of bacteria. As the dry feces per person average about an ounce, 30 grams, per day, this would mean that something like 8 grams of bacteria are given off daily by each individual and contributed to the sewage.[17] It must be remembered, of course, that only a small percentage are in a living condition estimated by some investigators at about 33×10^{12} per day per individual. The commonest species found is *B. coli,* though 31 species were detected in the sewage of Columbus, Ohio. Many of these bacteria come, of course, from other sources, soil, water organisms, excreta of animals, but the human intestine is responsible for many of them. The fate of many varieties of these bacteria in sewage is not known but, in general, few of them find this medium a favorable location for growth or even for existence. The presence of many organic substances in partially decomposed form, unavailable as food and probably somewhat poisonous, checks their growth and gradually kills them off.

The Function of the Bacteria.—The bacteria in the sewage serve to break it up and reduce the organic materials which are always very unstable to simpler ones. The organisms involved in the various processes are not known and they can be grouped together only according to the conditions under which they act. The destruction of the nitrogenous materials in sewage is so analogous to the same process as it occurs in the soil that it may be well to study it in some detail. In fact, more is known about the changes which occur in sewage than about similar processes in soil, since they have been studied with greater attention and many of the facts of ammonification and nitrification were worked out originally on sewage as has already been pointed out in Schlössing and Müntz's experiments.

Septic Tank.—It may be well, therefore, to look at a modern sewage-disposal plant where advantage is taken of bacterial activities on organic matter in suspension or in solution (Fig. 156). The sewage flows into a tank where it is held a short time in order to allow the heavier particles to settle out on the bottom of the tank, the lighter portions including the fats rising to the top. The liquid then passes into the *septic tank* proper where it follows a circuitous course among baffle boards which direct it first to one side and then to the other, in order to increase the distance traveled. A scum rises to the surface excluding the air. Sewage held in this tank for 18 to 24 hours comes out as an almost clear, slightly opalescent, almost odorless liquid.

What Has Occurred?—In the septic tank, the nitrogenous compounds of all sorts have been acted on by the anærobic bacteria growing in the depths of the liquid after the layer of scum has been established to seal over the surface. Some compounds are broken down by ærobes but putrefaction is largely anærobic; the anærobes performing this function more rapidly than the ærobic types. The process, therefore, is one of *ammonification;* the septic tank reduces the nitrogenous compounds to ammonium or ammonium compounds. The next process is to oxidize these compounds to nitrites and nitrates. For this reaction an abundant and constant supply of oxygen is required, such as cannot be supplied

in any container but must come from continuous access to the oxygen of the air. The best method is to trickle the liquid through sand, fine gravel, or cinders for a depth of 3 or 4 feet. The grains of sand become covered with a slimy growth of nitrite and nitrate bacteria and, as the liquid bathes them, they oxidize the ammonia into nitrates. These beds are, therefore, appropriately called "nitrifying beds."

This nitrifying process may occur in the soil and if sewage is used for irrigation this treatment in sand beds is not necessary. The water is run through pipes or trenches out over the fields to be irrigated.

Sludge.—In some English cities the sewage is passed through settling tanks to remove the sludge and in all cases through strainers to remove the floating material. This *sludge* which settles out is valuable as a fertilizer but difficult to dry and to dispose of after dried. It is sometimes pressed into dry cakes and used as fertilizer after being broken up, or, as a so-called "filler" for commercial fertilizers. Sludge itself is a too low-grade fertilizer to ship profitably but can be sold locally where it can be hauled directly to the farms. An enormous amount settles out of sewage; in Cambridge, England, for example, with a population of 55,000, the weekly production of dry sludge is 35 tons; in Boston, Massachusetts, the dry sludge amounts yearly to 67 tons for every thousand inhabitants although the majority of American cities produce less, averaging about 30 tons a year for each thousand of population.

FERTILIZER VALUE OF SOME ENGLISH SAMPLES OF SLUDGE[19]

	Chorley	Dorking	Kingston on Thames		Glasgow
Grit, insoluble	25.30	6.84	22.33	28.04	10.75
Lime	10.32	23.16	3.30	4.78	12.09
Phosphoric acid P_2O_5	.98	0.66	1.74	2.35	1.11
Nitrogen (total)	1.23	0.89	1.93	2.49	1.30
Potash			0.16		0.10

The variation in the amount of lime is a consequence of the use of lime in some of the cities as a settling agent added to the sewage. Potash is almost negligible in quantity but nitrogen and phosphoric acid are present in sufficient amounts to give sludge a small value as a fertilizer.

The commercial value of the sludge could be further increased by removing the sand and grit from the street washings by a short preliminary settling in a basin or trap.

The sludge of the city of Chicago has been estimated[31] to be worth at least $4 a ton as fertilizer while the cost of recovering would be only about $1.66 per ton. It is not recovered and the city of Chicago loses about $2,000 a day as a result. The city of New York empties at least $1,500,000 worth of fertilizer yearly into the rivers and bay.

The value of the sludge from English cities, used as a substitute for commercial fertilizer on wheat, has been studied in pot experiments.[19] With sewage sludge applied to land at the rate of 40 pounds of nitrogen to the acre, the yield of both straw and grain was increased 10 to 12 per cent above unmanured. The value was all

taken out in the first year, leaving nothing for the second year's crop. Lime must be in the sludge, or be added to it, to hasten the decomposition of the nitrogenous matter. Ten shillings ($2.50) a ton on the farm would be all that any sludge would be worth as a fertilizer.

Sewage Farming.—The principal objections raised against sewage farming are: (1) The lack of suitable land for such a method of disposal. Such land should be sandy, or at least sandy loam, close to the city, and not too expensive. (2) Difficulty of disposing of the products of sewage farms. Crops should be grown which are eaten by animals or with which sewage does not come in contact, such as wheat or grass. (3) The odor from such sewage-covered fields. This may be largely eliminated by using lime in the settling basins and by covering the sludge run on the fields with soil. (4) The health of the workers on such farms would be affected by the sewage. An English investigation made of all the cities using this method of disposal found not even a single case of sickness due to worker's living on a sewage farm. (5) The low value of the sewage as fertilizer. The sewage must be treated not only as fertilizer, but as irrigation water, and would be especially valuable in the West where the rainfall is not sufficient for the proper development of the crops.

Sewage-sick Land.—Land that has been too heavily dosed with sewage may become sour and heavy due to the soil pores being filled with fats. Such land must be allowed to remain free from sewage for some time, lime at the rate of 1½ to 2 tons to the acre applied, and the land plowed. The addition of lime assists in the nitrification since nitrifying organisms will not live in an acid soil.

Sewage Disposal in Country Homes.—Since the introduction of modern plumbing and running water into country homes, the problem of disposing of the sewage has arisen and rural engineers have devised systems that are based on the same principles as those used in cities.[35] The essential features are a septic tank with drainage from the tank into a stone pile or very open soil.

The septic tank (Fig. 156) should be large enough to hold all the drainage from the house for at least 24 hours and preferably 36 hours, if not longer. The size can be estimated by the water consumed; in general, about 10 to 11 cubic feet per person. During its retention in the tank much of the organic matter is partially destroyed. The solid material in the sewage either settles out or sinks to the bottom and the water which issues should be fairly clear, although only about 40 per cent of the necessary changes are as yet complete. In order to avoid all odors, such a tank must be covered, must be underground to prevent freezing, and must be constructed of strong concrete. The heavier material which settles out must occasionally be removed and provision must be made for such cleaning. The action should be as automatic as possible but, since it is absolutely impossible for any such apparatus to be entirely so, the necessary attention should be reduced to a minimum. The overflow can be regulated by a float and siphon but any piece of mechanism requires, occasionally, some care. The liquid which flows from the cesspool requires further bacterial treatment, such as can be given it only in gravelly soil where the air will have an oppor-

tunity to oxidize ammonium compounds into nitrites and nitrates. This part of the treatment is as important as the septic tank. The water from the tank may be conducted through 4-inch tile set about ¼ inch apart, not less than 35 feet being used per person on light sandy soil or 60 feet on heavy land. The tile should not have a slope of more than 1 inch in 50 feet, or the water will all rush to the lower end and clog the opening and soil. At least 450 to 500 square feet should be allowed for each person connected with the system.

The Organisms of Sewage and Septic Tanks.—The organisms which occur in sewage and in sewage disposal plants are very difficult to study, and they remain to a large extent one of the dark continents of bacteriology. The carbohydrates, fats, and proteins go into the septic tank or the filter beds or soil; the simpler compounds are evolved

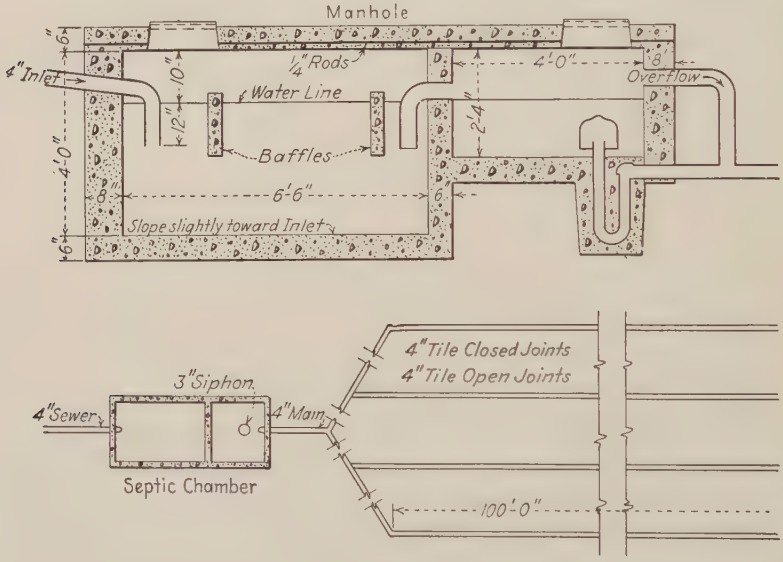

Fig. 156.—Septic tank devised for country homes where no sewer connection is available. (*After Trullinger.*)

but the particular organisms or even the types of organisms which predominate in the various processes can, to a large extent, be guessed only. The whole problem is very complex, with many organisms and many reactions; the by-products of one reaction are used as the material for other reactions as soon as formed and little trace can be obtained of the intermediate products.

Miss Hotchkiss[12] attempted to follow these processes in the septic tank and on the sprinkling beds in order to determine the predominant types at various stages of the destruction of the materials in sewage. As the results of a year's test of the septic tanks of the Imhoff type, she arrived at the conclusion that nitrate reducers were the predominant group of bacteria but that hydrogen-sulphide producers (the sulphur from protein destruction) and albumen digesters were next in importance. Nitrifying and sulphur-oxidizing bacteria were always found in the tank, although a

chemical analysis showed no nitrites or nitrates. The number of organisms which produced hydrogen sulphide seemed to be smaller just before the sewage passed out of the tank, as the very first step in the breaking up of proteins results in the formation of this gas. On the sprinkling beds, the oxidizing types were predominant, as was to be expected, and proteoclastic forms, fewer in numbers, as the proteins had been largely destroyed before they arrived at this stage.

THE FILTRATION OF DRINKING WATER

Bacteria and Drinking Water.—Large cities are finding the problem of pure water for drinking purposes increasingly difficult of solution, as the country becomes more densely settled, and natural waters are contaminated with the sewage. New York has gone miles into the Catskills and bought up great tracts of land in order to protect, as far as possible, her future water supply from contamination. The same problem confronted ancient Rome, where it was solved by bringing water over 30 miles from the Sabine Mountains across the Campagna in great stone aqueducts to supply the city fountains. Not many cities of the modern world can afford to carry the water from such distant sources, as does the city of Vienna where a long aqueduct brings an adequate supply from the Semmering Pass in the high Dolomite Alps, a distance of over 60 miles. Almost all cities depend on filtering a water which is relatively unfit for drinking purposes in the raw state—but the purer the water taken, the less work there is for the filters, and the less danger after filtration.

An example of the possibilities of using impure water for drinking purposes is given by the city of Burlington, Vermont, where the supply is derived from the same lake into which the city sewage is poured.[20] Before 1894, with water pumped directly from the water front into the city mains, the intake being only ¾ mile distant from the outlet of the sewer, Burlington was much afflicted with diarrhea and occasional outbreaks of typhoid fever. It was determined to remedy the situation by bringing water from the best portion of Lake Champlain (Fig. 159). Professor Sedgwick, the best American expert on water bacteriology, located the intake after determining the bacterial contamination of the water along the lake front for some distance up and down the city frontage. The problem was further complicated by local conditions: (1) by the sewage which was emptied into the lake about 10 miles north by the Winooski River, a fairly large stream filled with the sewage of four or five small towns, and (2) by the impossiblity of extending the pipes directly out into the lake as the shore shelf disappears almost at once and the bottom pitches to a depth of about 100 feet. As the water to the south was also badly contaminated, a point north of the city and midway between the outlet of the Burlington sewer and that of the Winooski River gave the purest water. Even at this distance, sewage is carried to the intake by the wind; so that when the south wind blows, the Burlington sewage drifts north; with a north wind it receives Winooski River contamination. The typhoid rate in 1893, the year before the position of the intake was changed, was 3.26 per 10,000 inhabitants while in 1895 it had dropped to 0.32 in 10,000. The system was further improved in 1908 by the installation of rapid-sand filters which remove about 98 per cent of the 1,000 bacteria (approximately) in the raw water. So that, although, as Prof. Sedgwick once said, Burlington is famous as the city that drinks its own sewage, the water

supplied from the city taps is excellent in quality and harmless, due to the sedimentation, filtration, and chemical treatment which it receives.

For Household Purposes.—The filtering of small quantities of water for drinking purposes in a household can be effected by a Berkefeld clay filter attached directly to the faucet. The force of the water drives it through the filter, but even then it will only drop, or flow, a slow trickle insufficient for any large quantity. Unbaked clay filters contain pores which vary in size from those in the coarse Berkefeld clay filter

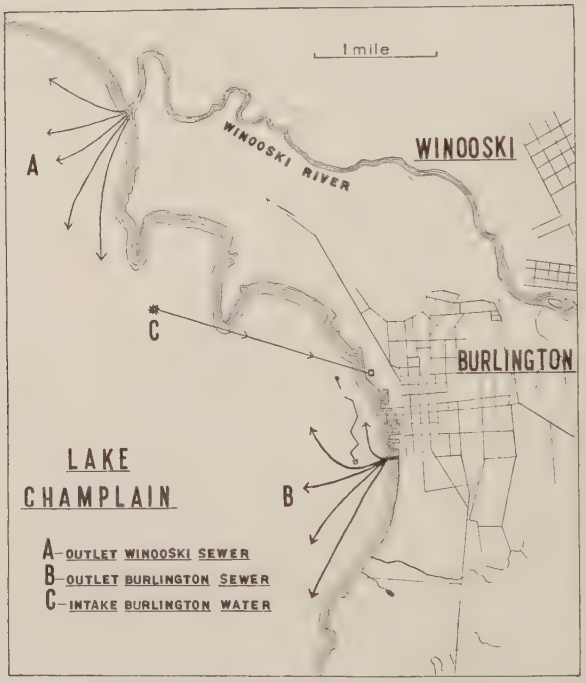

FIG. 157.—Spread of sewage in a large lake; obtaining pure drinking water where the sewage flows into the same body of water as that from which the drinking supply is derived.

to those of the very fine Chamberland or Pasteur-Chamberland type (see p. 17). The rate of flow is necessarily retarded by the small size of the capillaries but can never be very rapid if all the bacteria are to be excluded. The bacteria gradually find their way deeper and deeper into the filter until finally they come through in numbers. The only practical method of cleaning is to bake the filter in a chemical oven where the organic material is reduced to an ash.

The Pasteur-Chamberland filter is too slow in operation for the household, but it is very valuable for laboratory purposes where bacteria-free substances are desired.

For City Water Supplies.—The problem of securing a safe drinking water for a large city is a difficult one to solve when the only water that

can be found within a reasonable distance is highly contaminated with sewage. The problem in Burlington, Vermont, just cited, perhaps is not as acute as it is in some mid-western cities where the only available supply is a muddy and polluted stream, such as the Missouri, Ohio, or smaller rivers. This water must be rendered clear, palatable, and free from dangerous bacteria in quantities that will average about 250 gallons per inhabitant per day. The water engineer may use (1) *sedimentation*, (2) *coagulation*, (3) *filtration*, (4) *chlorination*.

Sedimentation.—This treatment is especially valuable where turbid river water must be used. The small particles of soil settle out of water held in a quiet basin and carry with them a considerable proportion of the bacteria. London has used this method for years on the dirty water of the Thames River, reducing, by a 14-day stay in a settling basin, the number of bacteria from 4,465 to 362. Even a few hours' sedimentation removes the larger soil particles and many of the organisms. The finer colloidal clay is not removed in this short time but, without this preliminary treatment, filtration is impossible with many cloudy river or lake waters.

Filtration.—Two methods of sand filtration are in common use in this country and abroad, *slow-sand filtration* and *rapid-sand filtration*. The difference is expressed in the names, since slow-sand filtration permits the passage of only 6,000,000 to 8,000,000 gallons per day per acre of filters while rapid-sand filters care for 100,000,000 to 125,000,000 gallons. The methods used are somewhat different for the two types and as the slow-sand filter is the older one, it will be discussed first.

FIG. 158.—Section of Cincinnati sand filter; the different layers held apart by wire screens.

The slow-sand filter consists of a layer of sand and gravel about 3 to 4 feet in thickness through which the water is passed and collected in pipes (Fig. 158). The sand and gravel do no filtering but support on top of the sand a colloidal living deposit through which the bacteria cannot find their way (Fig. 159). Since at least 2 days are required to form this deposit, a proportion of the filters must always be out of operation, so far as filtering is concerned. In spite of this disadvantage, some of the largest cities of the country have adopted slow-sand filtration: Washington, District of Columbia, Albany, New York, and Philadelphia, Pennsylvania. The plant at Washington, District of Columbia, contains 29 filters, each 1 acre in extent, and cost $3,300,000 to install. Its efficiency is shown by the fact that it removes 98 to 100 per cent of the bacteria at a cost of only $2.41 to $2.77 per million gallons filtered. The Philadelphia filter removes all but 35 bacteria per cubic centimeter with an intake water containing 1,100 to 6,600.

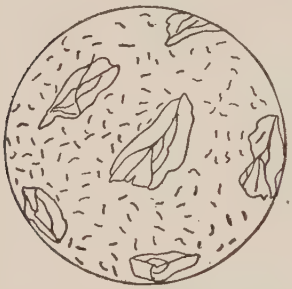

FIG. 159.—Largest common bacteria (*Megatherium*) and smallest sand particles drawn to same scale (× 50). (*From Flinn, Weston, and Bogert. "Waterworks Handbook."*)

The principal objections to the slow-sand filter are (1) The great acreage required. (2) The filters clog quickly and must be cleaned often if turbid river water is used. (3) The great supply of sand that must be handled either by obtaining new sand or cleaning the old sand. Rapid-sand filtration is slightly more expensive, costing in 1912, for the cities that were investigated, $4.04 per million gallons against $2.68 for the slow-sand process, but has certain advantages that make it the only feasible type for certain waters. Turbid river water such as is used by St. Louis and Cincinnati, can be handled successfully only by sedimentation and a previous *coagulation* by means of some chemical.

Rapid-sand Filtration.—This type of filter was first used to secure pure water for pulp mills where the slightest sediment stains the paper. The discovery was made in 1897 that it would serve also to remove the bacteria, when properly handled, being just about as efficient as the slow-sand filter and removing 97.5 to 98.5 per cent of the bacteria.

In the use of the rapid-sand filter, it is not feasible to obtain the colloidal layer on the sand *by growth*, so colloids must be supplied *chemically*. The chemical added is either alum or ferrous sulphate. With alum, the reaction of the water must be hard, *i.e.*, calcium or magnesium carbonate must be present to react with it, or they must be added, since the changes which should occur are expressed by the following reaction:

$$Al_2(SO_4)_3 + 3CaCO_3 + 3H_2O = 3CaSO_4 + 2Al(OH_3) + 3CO_2$$

Aluminium hydroxid is insoluble and comes down as a colloidal white precipitate. The alkalinity of the water is reduced and theoretically, all of the alum should be removed, since the colloid which it forms

remains on the surface of the sand filter when the water is passed through the sand. The amount of alum added depends on the condition (turbidity) of the water, averaging from 1 to 5 grains per gallon.

The water coming from the intake pipe is conducted to the settling basin, where the coarser particles are removed, and then to the coagulating basin where the alum solution is added and coagulation occurs. Some of the proteins in the water are gathered into the colloidal mass and much of the coloring matter which the water has acquired from the decaying plant tissue along streams and in lowlands is absorbed by it. It is now passed out over a sand filter similar to that used for the slow-sand process, and in a few minutes the colloidal alumina has formed a strainer on the surface and the filter is in active operation. Some method must be provided for freeing the sand from the colloidal deposit, the one usually adopted being to reverse the filter and run off the dirty water. In order to prevent the mixing of the sand and gravel, a fine copper screen is placed on top of the layer of gravel.

Chlorine Treatment of Water.—The filtration process can be further supplemented, where the water contains numerous bacteria, by adding to the water some chemical, injurious to the bacteria, but not in sufficient quantity to injure the health of persons drinking the water or to give a taste. Chlorine is now usually added as a gas from tanks in which it is kept in a liquid condition. The amount added is very small,[6] 0.2 to 0.5 part in a million being usually sufficient to disinfect water. If more is added, the taste becomes noticeable, the average person being able to detect 0.6 part. The cost of chlorine gas varies between 10 and 15 cents per pound so with only 2 to 5 pounds per 1,000,000 gallons, the cost is very small.

The intestinal organisms seem to be especially susceptible to the action of the chlorine.

In Cincinnati in 1915, sedimentation removed 81 per cent of the bacteria, coagulation and filtration (rapid-sand) 96 per cent, and disinfection with chlorine 71 per cent. With all these methods combined, the numbers surviving are very small. The typhoid death rate has been just about halved where chlorine has been added to the water. Baltimore reduced the rate per 100,000 from 35.2 to 22.8; Cleveland, 35.2 to 10.0; Kansas City, 42.5 to 20.0; and Omaha, 22.5 to 11.8.[5,29]

THE EXAMINATION OF WATER FOR DRINKING PURPOSES

Water intended for human consumption is subjected to very careful scrutiny both as to its chemical and bacteriological properties; the chemist can tell much but the bacteriologist can add much more.

The Chemical Examination of Water.—The chemist examines a water for its color, odor, and appearance, as these properties are of importance from the aesthetic side, although they may give little indication as to

the possibility of its infection with disease-producing bacteria. More important for sewage detection are the chlorine and organic content.

The *chlorine* in water is present as chloride of sodium. This chloride may come from the rocks and soil, from the indriving ocean spray, or from the salt which human beings use in such large quantities and which finds its way eventually into human waste products. Each region has a *normal chlorine content* for its waters but if this amount is much exceeded, the water may have received some human excreta containing, as is usual, a very high percentage of salt.

The organic content, especially the form of the compounds of nitrogen, also indicates whether water has been polluted or not. The nitrogen compounds may be albuminoid nitrogen, ammonia nitrogen, nitrites, and nitrates. The presence of much *albuminoid nitrogen* indicates that the organic material is still largely undecomposed and that the contamination was recent and near to the source of the sample. Ammonia nitrogen, formed when organic compounds are destroyed by bacterial action, means pollution a little more remote than that of albuminoid nitrogen. Much nitrogen in the form of nitrites shows that the water has been contaminated with organic matter but that the bacteria have destroyed it and that it has been partially oxidized, but that the water is still doubtful; if it is present as nitrates, however, the possibility is small that the pollutions are a danger, as they are too remote. From an analysis of the nitrogen content the chemist can tell the nearness or remoteness of pollution with human or animal excreta.

The Bacteriological Examination of Water.—The bacteriologist adds, to the above account, two important facts: (1) The number of bacteria present in the water, determined by plating out samples of the water on agar, incubating for 24 hours, and counting the colonies. (2) The presence of sewage organisms, indicated by their growth and gas formation in the fermentation tubes, as described for *B. coli*. Of the two tests, the latter is the more important, as the presence of *B. coli* is to be always regarded as a matter of suspicion since, if this common intestinal organism occurs, *B. typhosus* may also be found. Although all gas-forming organisms may not be of fecal origin, the gas test is a reliable one for the sanitary quality of water.[39]

The methods for water examination in this country have been very thoroughly standardized by committees of bacteriologists interested in this phase of bacteriological work, and the whole procedure brought together as the "Standard Methods for the Examination of Water and Sewage."[34]

Bacterial Standards in Drinking Water.—The careful selection and filtration of drinking water has two objects in view: (1) to supply water with a low-bacterial content and (2) to free it from all sewage organisms. Water with a high-bacterial count would contain 200 or more per cubic centimeter; the sewage contamination is measured by the abundance of *B. coli*. Drinking water should not contain more than 100 bacteria per cubic centimeter and the American standard is even lower than this, 50 per cubic centimeter, and it should not contain a single *B. coli* in a cubic centimeter.

The very high typhoid death rate of 20 years ago in American cities has been cut to about one-third by proper filtration methods. Lowell, Massachusetts, reduced the rate from 13.9 per 100,000 to 1.7; Pittsburgh, Pennsylvania, from 65 to 3.9; Paterson, New Jersey, from 19 to 3.6; Reading, Pennsylvania, from 42 to 5.4; and Jersey City, New Jersey, from 12.6 to 2.6. Fifty-seven large American cities in 1910 had a typhoid death rate of 19.59 per 100,000 population, while in 1924, this had been reduced to 3.07 per 100,000.[16,18]

The total population of the cities of the United States which make returns of vital statistics is 34,230,000 and 48 per cent of this number, or 16,500,000, were supplied with filtered drinking water. This number is 17 per cent of the total population of the United States.[22]

BAD TASTES AND ODORS IN DRINKING WATER

Water from ponds or lakes acquires, at certain seasons of the year, tastes and odors which are offensive and render it unpalatable. Such

FIG. 160.—Some algæ, especially blue-greens found in water: 1, *Oscillatoria limosa;* 2, *Oscillatoria irrigua;* 3, *Lyngbya major;* 4, *Lyngbya æruginco-cærulea;* 5, *Anabæna inequalis;* 6, *Cylindrospermum stagnale;* 7, *Nostoc sphæricum;* 8, *Nostoc paludosum;* 9, *Clathrocystis;* 10, *Asterionella.* (*After West, except* 9 *and* 10 *after Moore.*)

water is usually at once a matter of comment and is popularly believed to be infected with harmful bacteria growing in it. As a matter of fact, however, bacteria are not responsible for the real contamination, although they may be for the release of the offensive products. The source of the bad tastes and odors are algæ which, growing during the summer months, are killed by the hot sun of August and decay in the water. A number of varieties of algæ are involved; one species in one epidemic and an entirely different series in another. The commonest group to be found under such conditions is the blue-green algæ, although true green algæ and diatoms are also involved.

The Odors.—The odors have been described as "pig-pen," "sulphurous," "fishy," "moldy," "musty," "grassy," and "indescribably suffocating"; all very indefinite adjectives, but descriptive terms for olfactory sensations are not clear and no standard

of comparison exists. These odors with the accompanying tastes are, in the majority of cases, due to blue-green algæ (Fig. 160).

The Blue-green Algæ.—The *Schizophyceæ*, known also as the *Cyanophyceæ*, with blue-green coloring matter, are unlike the ordinary green or yellow-green algæ in general appearance, coloring matter, structure, and reproduction. The green tint is most nearly comparable to that of the needles of a dark fir tree. The coloring matter, *phycocyanin*, is physiologically similar to the chlorophyl in that it is able to transform CO_2 and water into starch but is chemically different. It is united with chlorophyl and a third coloring matter in the cells of the blue greens. It is even doubtful if the green color is the same in all blue-green algæ, since blue-green, blue, and blue-violet pigments, or modifications of a single pigment, have been isolated chemically. These coloring matters are held in the outer layer of the cell in a sheath surrounding a denser inner portion.

The blue greens are common in stagnant water in ponds or reservoirs, especially in the late summer; others form the green slime which appears at all seasons in greenhouses, covering the soil and pots. Some of them are single cells, living independently or in colonies of irregular size and shape. Many form long filaments with short cylindrical cells, long cylinders, beads, or spheres. In many species the cell rows are occasionally broken by larger, apparently empty cells, "heterocysts" (heter = unlike, cyst = cell), the function of which is unknown, although they are supposed to play some rôle in reproduction.

The cells lengthen as they grow; the row of cells in a filament are all about the same diameter, and growth is limited to increase in one dimension. They divide by the ingrowth of a new wall midway across the lengthened cell. This wall, when it reaches the interior of the cells, splits into two plates which free the two daughter cells by the process known as "fission."

As long as these organisms are actively growing, making use of the salts dissolved in the water and the carbon dioxide of the air, they are entirely inoffensive, but when they have been killed by the hot sun, their bodies decay and, in the breaking down of the protoplasm, fat droplets develop, escape from the ruptured cells, and give a taste and an odor to the water. These oils are very small in amount but essential oils, such as peppermint, have been found to produce detectable tastes when present in the proportion of only 1 part of the oil in 5,000,000 of water.[38]

The products formed in this decomposition of the body of the blue greens are not necessarily harmful in any way, although Arthur in 1883 records a case where the use of water, from a small lake covered by a scum of blue-green algæ, by cattle, horses, and sheep caused death. The organism was identified as *Rivularia fluitans*. The majority of the blue greens would not injure water further than to produce bad tastes and odors.

The Water Bloom on Ponds.—Some species of blue-green algæ are responsible for the growth on the surface of quiet waters, the "water

bloom" which appears during the summer months and is associated with "turning of the meres" in England. The commonest form of blue green to appear in such cases is a spherical species known as Cœlosphærium, although various species of Anabæna also have been observed.[26] The most complete study of the occurrence of the blue-green algæ in this country for a succession of years has been made of the waters of Jamaica Pond near Boston by Isabel F. Hyams and Ellen H. Richards[13,14,15], who followed the development of the blue green, *Oscillatoria prolifica*, there between the years 1887 and 1904 with regard to its life history, chemical composition, and coloring matters.

Freeing Ponds and Reservoirs of Algæ.—City reservoirs open to the direct sunlight often develop very large crops of algæ during the latter part of the summer. If such reservoirs can be covered, the growth can be prevented, owing to the absence of sunlight, but such a method is very expensive and, in general, unnecessary, so many municipalities have had recourse to chemical means for destroying plant growth. These chemical treatments consist in the dissolving in the water of very small traces of copper sulphate, usually about 1 part in 5,000,000 parts of water. The algæ are even more sensitive than the fungi and the bacteria to the action of the copper compounds and in a few days die from the effects of the small traces absorbed. Their bodies then decay and give the water a peculiar taste, usually popularly attributed to the use of the copper sulphate, but really from the decomposition products of the algæ. Usually one treatment is sufficient for a season, so that cheapness and ease of application have made this method a regular procedure in the cities of the southern part of the United States where the growth of algæ in the reservoirs has been particularly objectionable. The use of this small amount of copper sulphate is not sufficient to injure the health of users of the water but may cause the death of fish, if the reservoirs happen to be stocked. Fish are killed by approximately the same amounts as algæ so that the percentage of copper sulphate must be very accurately calculated.[25]

Algæ Frozen in Ice.—Algæ float on the surface of the water buoyed up by the formation of gases. If the growth occurs late in the fall, during October or early November, many of them may become caught in ice and remain there until the following spring. Such a case was investigated by Olive[27] in a small Wisconsin lake, and similar instances are known over the northern states where natural ice is cut from ponds and lakes. The alga does no damage to the ice during the time it is held frozen, but after its release, when the ice melts, it may give rise to odors similar to those produced in water.

ICE

Bacteria in Ice.—Bacteria do not thrive in ice, lack of both food and water being responsible for this condition. The organisms, held in one position, can absorb neither food nor water from their surround-

ings. The consequence is that they die off rapidly during the first few days that they are imprisoned in ice, the more susceptible individuals probably succumbing in a few hours. The organism in ice upon which interest focuses is the bacillus of typhoid fever; since this disease is water spread, it would seem possible that it might also be ice spread. Prudden, quoted by the Franklands,[9] froze this organism in an ice machine and kept it for 103 days, taking out samples at times to determine its viability and the number of organisms. After 11 days, 1,000,000 per cubic centimeter were still present, after 77 days, 72,000, and at the end of the 103 days, 7,000. If, however, instead of being subjected to a steady low temperature of 14.30°F., the cultures were frozen and thawed a number of times, the alternation was very deadly to the organisms. Typhoid bacteria frozen for 24 hours but interrupted by three thawings were reduced from 40,000 at the begininng to 90 at the end of the day, with all dead at the end of 3 days.

Experiments by Sedgwick and Winslow[33] on the typhoid bacillus gave somewhat similar results. A large number of tubes of water inoculated with this bacillus, three strains being used, were frozen and the numbers determined at different times; 20 tubes being used in each case in order to get an average. From 30 to 60 per cent were killed during the first hour of freezing, the numbers after this time slowly diminishing for 2 weeks when the constant value of 99 per cent was attained and the remaining 1 per cent remained nearly constant. At the end of 12 weeks, a few were still living, some strains used being more resistant than others. *Freezing and thawing* had also a very detrimental effect on the *B. typhosus* as also did cooling (not freezing) in water. The latter point is of interest as it shows the susceptibility of human internal parasites to low temperature even above that of ice. Experimental work showed that over 90 per cent of the bacteria in water are not frozen into the ice at all but are physically excluded in the freezing process.

Similar results were obtained by Reudiger,[30] who added typhoid bacilli or colon bacilli to tubes of water, frozen, and kept in a shed at winter temperatures, the numbers being determined for 46 days with the following results:

Duration of Life of Typhoid Bacilli and Colon Bacilli in Ice Kept in a Shed in Winter Weather

	Typhoid bacilli, number of typhoid bacilli in ice	Percentage of typhoid bacilli living	Colon bacilli, number of colon bacilli in ice	Percentage of colon bacilli living
Before freezing....	214,000,000	100	269,000,000	100
Frozen 8 days...	145,000	0.067	7,000,000	3
Frozen 14 days...	54,000	0.025	5,000,000	1.85
Frozen 22 days...	220	0.001	1,020,000	0.38
Frozen 31 days...	0	0.		
Frozen 36 days...	.....	.....	10,700	0.0039
Frozen 45 days...	.....	.....	1,950	0.00072
Frozen 46 days...	.....	.....	0	0

These figures indicate that the colon organism is somewhat more resistant than the typhoid bacilli to freezing and able to survive under unfavorable conditions.

In view of the above results, the possibility would be remote that ice could serve as the carrier of typhoid fever and in fact we have only one record of such an epidemic, from ice stored 5 to 7 months but heavily polluted.

If the ice is cut where the water is free from sewage and from surface contamination, a large part of the bacteria will be physically excluded as the water freezes, a very large portion of the typhoid organisms will perish very shortly, and if the ice is stored for a few months the danger from typhoid fever from its use is almost negligible. *If ice is clean and transparent with no visible dirt, it is usually safe to add to drinking water.*

It must be noted that the death of microorganisms, as the result of exposure to cold, imprisoned in ice is not corroborated by the results obtained by Butjagin,[2] who left cultures of various bacteria, such as *V. choleræ asiaticæ*, *B. coli*, *B. typhosus*, *B. typhi*, *B. paratyphi*, *B. prodigiosus*, *B. anthracis*, *and B. subtilis*, exposed under about 2 meters of snow in one set of experiments, and enclosed in a box outside the laboratory window in another, during the rigorous cold of a Siberian winter when the thermometer went down one time to a temperature of −44.8°C., and found that they nearly all survived the 3-month treatment. A few of them, such as the organism of cholera, succumbed. Similar results were obtained by Bartram[1] who exposed cultures of various non-pathogenic bacteria during a Vermont winter and found that some still survived and produced typical growth.

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CHAPTER XXXV

THE BACTERIOLOGY OF MILK AND ITS PRODUCTS

Milk is secreted from the glands which make up the bulk of the udder of the cow, these glands or follicles leading by a tube into larger ducts (Fig. 161). After secretion, the milk collects in a large reservoir, the milk cistern which contains about ½ a pint.

FIG. 161.—Milk secretion, milk, and lactic acid organism (*Strept. lactis*): *A*, section of udder of cow showing milk cistern and ducts; *B*, lobule of gland filled with milk globules; *D*, fat globules, Holstein; *E*, lactic-acid' streptococci with one streptococcic chain; *C*, fat globules, Jersey. (*A, after Moore and Ward; B, from Fleming's "Veterinary Obstetrics;" C to E, original.*) *C* to *E* × 800.

Milk is a complete food for many bacteria as well as for human beings, since all the necessary constituents for growth are present.

Composition of Cow's Milk	Per Cent
Water	87.17
Fat	3.69
Casein	3.02
Albumin	0.53
Sugar	4.88
Ash	0.71

The percentage of fat in milk varies with different animals and breeds. The fat is a mixture of esters of glycerol with volatile or non-volatile fatty acids, such as oleic, stearic, palmitic, myristic, butyric, lauric, caproic, etc., and is not a single chemical.

Milk sugar (lactose), a carbohydrate with the same chemical composition as cane sugar but with very different properties, is split by many species of bacteria into lactic acid, and a few of the yeasts are able to utilize it to produce alcohol. The ash consists of phosphates of lime and potash, chlorides of potash and soda, and small amounts of the phosphates of iron and magnesia.

Bacteria in the Cow's Udder.—The popular impression that the milkman is responsible for all of the bacteria found in the milk, introduced there by his careless handling, by dirty containers, or by failure to cool the milk properly is incorrect. The milk cistern of the cow's udder contains milk which has a rather high count of bacteria (Fig. 161). The upper portions of the udder of the normal cow are not so badly infected unless the cow has been diseased, when many millions of bacteria are to be found there also. This means that, in milking, the milk which comes first (fore milk) is usually very much higher in bacterial count than that which comes later. The last milk, (strippings) should have the lowest count, although this is not always true.

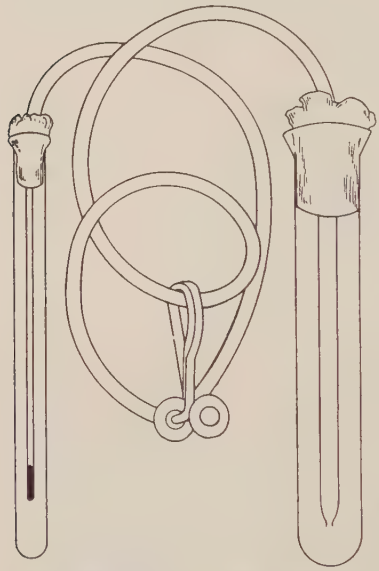

FIG. 162.—Milking tube by which it is claimed that sterile milk can be drawn from the upper portion of the udder. (*After Swithinbank and Newman.*)

Swithinbank and Newman,[35] using of germicides on the outside of the flanks, belly, and teats of the cow, drew with a sterile, long, slender silver-plated milking tube (Fig. 162), rubber tube, and test tube, sterile milk from the middle lactiferous ducts (see also Breed).[24]

Harrison and Savage,[19] on the other hand, claim that even with sterilized milking tubes and thorough disinfection of the udder they were able to get sterile milk only occasionally. The organisms which they isolated from the udder formed white or yellow pigmented colonies, some alkali, others acid formers. These organisms are found in the upper portions of the udder near the gland tissue. The results are confirmed by Breed,[3] who estimates an average of 500 per cubic centimeter in the normal udder, mostly micrococci.

Milk from individual cows and from the various teats has been examined in order to get some idea of the udder contamination to be expected under the very best controlled conditions,[20] from supposedly healthy cows:

Name	Days sampled	Average per cubic centimeter	Maximum per cubic centimeter	Minimum per cubic centimeter
Brownie	61	31,000	305,000	1,700
Dorine	33	191,000	3,500,000	2,500
Merney	27	810	4,250	50

As the cow ages, the bacterial content frequently increases, a fact to be expected when it is remembered that a large percentage of cows, at one time or another, have inflammation of the udder, and that an infection occurs from which they probably never entirely recover.[9]

Milk in the udder or when first drawn has a germicidal effect for some hours on certain bacteria, although apparently not on the normal micrococci of the milk cistern. Russell and Hastings[33] injected organisms, such as *Streptococcus lactis* and *Bacillus prodigiosus*, and Copeland and Olson,[9] such as *Bacillus œrogenes*, into the udder of cows and found that they disappear in a few days, owing to the germicidal action of the milk. This same power of the milk causes the number of bacteria in a sample to decrease sharply immediately after milk is drawn. The time during which the germicidal action is effective is longer when the milk is held at low temperatures, so some workers regard this stage as a readjustment period; some less hardy species, as well as many individuals among all udder species, die and the survivors become acclimated to the new conditions.

Leucocytes.—Milk always contains leucocytes (leuco = white, cyte = cell) derived from the blood stream. A high-leucocyte content means inflammation, which is always accompanied by high-bacterial numbers, and by the invasion of thousands of these cells from the blood stream. Normal milk always has a variable number, so that it is difficult to establish a leucocyte standard. Apparently normal animals may have often as high as 2,191,000 per cubic centimeter and as low as 1,250 per cubic centimeter, while diseased animals range in numbers between 4,232,000 and 7,500 per cubic centimeter. The presence of red blood corpuscles indicates a serious condition of the udder, and milk containing them is unfit for use.

Other Bacteria in Milk.—The great majority of bacteria which occur in milk, however, are introduced while the milk is being drawn or after it is drawn, as it is doubtful if many udder organisms survive for a very long time, although they may have an effect on the milk before they die or before their numbers become relatively small, as compared with those of the rapidly growing lactic-acid species. In addition to the udder contributions to the bacterial content, organisms find their way into milk: (1) from the milking machine with its tubes and pail, milk pails, milk cans, and bottles; (2) from the body of the cow herself in the form of bits of dust and dung; (3) from the air of the room in which the milking is done.

The utensils are the most important of these sources of contamination. The organisms introduced by the utensils are largely the sour-milk bacteria which may grow in carelessly washed milking-machine tubes, or in the crevices and corners of the milk pails. These organisms, protected by the film of milk in which they have dried, will live for some days in a quiescent condition, to be revived by the addition of fresh milk. Modern milking machines frequently add more bacteria than did open pails, since the long rubber tubes are difficult to keep clean and introduce many bacteria. Fluorescent and water types also come from poorly washed or scalded utensils.

Small pieces of dung or particles of dust and dirt from the hairs may fall into the milk, if the milking is done into open pails. The organisms which occur normally in

cow manure, such as *B. coli* and *B. œrogenes*, are both strong gas producers. The organisms which come from the air of the room are of the soil-bacteria type and include the ordinary soil organisms, the potato and hay bacillus, as well as other spore formers, many of which are digesting (peptonizing) types. If they predominate, a sweet rennet is formed and the coagulum is digested into a straw-colored, slimy liquid. Since the lactic-acid species usually become the predominating types, milk will normally "sour" if kept at ordinary temperatures. The lactic-acid types probably come from the utensils, although many species which form a small percentage of acid may be introduced from manure. The old idea that dirty barn air was responsible for the bacteria in milk has been discarded to quite an extent, although some bacteria fall into the milk pails if the old type of open pail is used.

Straining the milk through several layers of cloth in order to remove visible dirt does not lessen the number of bacteria in the milk. The straining process breaks up the clumps of organisms and distributes the individuals throughout the milk. A similar breaking up of the clumps of bacteria is accomplished by the mechanical clarifier. The slime removed contains large number of organisms but the milk contains practically as many bacteria as it did before clarification. Some investigators find an increase of from 13 to 260 per cent while others report occasional small losses.[28]

Effects of Keeping on the Bacteria.—The temperature at which milk is kept materially influences the length of time before souring. If the milk producer wishes to put on the market a milk with the lowest possible bacterial count, the initial bacterial content must be small, the milk kept at a low temperature, and the time between milking and using reduced to a minimum. Low temperatures influence the growth of certain types of organisms more than others, so that if milk is kept chilled, the bacterial flora is altered. The soil and hay organisms grow well at relatively low temperatures while the lactic-acid germs, being accustomed to the temperature of the body of the animal (37°C.), do not divide as rapidly. The growth of *all* organisms is retarded by very low temperatures, but some species are more susceptible than others, so that the ratios change with the temperature. The fact must be recalled that milk is a very perishable product, one which provides food for almost any of the bacteria, and that if one organism does not induce changes in it, another will, before many days. At the present time, when the large eastern American cities are deriving their milk from great distances, the problem has become very important. New York City gets milk not only from Vermont and New York but from Canada and as far west as Buffalo. Since the milk is frequently two days old before it is consumed, the original number of bacteria should not be high and a low temperature should be maintained by the use of ice, especially during the warm weather. As these cities continue to increase in size, the problem will become still more difficult of solution, since the milk will have to be brought from still more remote farms.

Milk warms after it is drawn but the warming is not due to the destruction of the lactose to form lactic acid,[32] as this fermentation plays only a minor rôle in heat production. This acid formation represents less than half the total amount of conversion of energy in the milk during this time. Lactic-acid fermentation is the only source of energy of this organism, and normal respiration, with oxygen from the air, will not stop this process even in well-aerated media.[25]

THE NORMAL CHANGES IN MILK

1. *The germicidal stage* in milk is the period immediately after milking during which the numbers of bacteria decrease or fail to increase in the milk. The length of this period is dependent on the temperatures as the following table (Hunziker) shows:

Number of bacteria in freshly drawn milk	Temperature of milk	Number after 3 hours	Number after 6 hours	Number after 9 hours	Number after 12 hours	Number after 15 hours	Number after 24 hours	Number after 32 hours	Number after 48 hours
1,212	40°	1,080	1,220	1,040	1,020	1,120	1,360	1,640	400
	55°	1,260	1,400	1,500	1,462	1,360	1,080	3,500	17,740
	70°	1,000	1,340	1,860	3,460	3,460	64,000	800,000	

Milk seems to contain an enzyme which destroys organisms, its effect being limited to the first day after the milk is drawn and to unheated milk.[8]

2. *The souring stage* follows in the next 24 hours with the rapid increase in lactic-acid organisms and enough acid to coagulate the casein (Fig. 105).

3. *The neutralization stage* is a long one at ordinary temperature, since the growth of the *Oidium lactis* yeasts and molds which destroy the lactic acid is slow. At the end of several weeks, the acidity may become lowered to the point of decomposition.

4. *The decomposition stage* is introduced from the growth of the bacteria which gradually digest the proteins of the milk and finally reduce it to an opalescent liquid. The growth of these digesting types is suppressed during the period of high acidity, but as soon as the fungi have destroyed the acid, they have a chance to grow.

PASTEURIZATION

Pasteurization is the process of heating milk to kill all non-spore forming bacteria. Usually from 95 to 99 per cent of the microorganisms are killed; so the process is now frequently used to reduce the bacterial content, including that of the common pathogenic species.

Heating induces changes, not only in the bacterial content, but also in the proteins in the milk itself, but if the temperature is not held too long nor too high, the latter are reduced to a minimum. The problem,

therefore, is to destroy the bacteria as far as possible without changing the taste or chemical properties of the milk. The thermal death point of the ordinary milk bacteria has been determined, a temperature which usually lies with the majority of the milk bacteria between 55 to 60°C., so that 65°C. for 20 minutes is sufficient to destroy them, one of the most resistant being the tuberculosis bacillus with a thermal death point above 60°C.

The two common methods employed for milk pasteurization are the "flash" and "holder" systems. During the "flash" process, the milk is heated to 160 to 175°F. and immediately cooled to 50°F. or less. In the "holder" process the milk is heated to 140 to 145°F., held for 30 minutes, and then cooled off to 50°F. or less. Certain pasteurizing machines have been sold which do not meet the requirements of pasteurization because all of the milk is not exposed to the desired temperatures. Recently, electrical pasteurization has been introduced and some boards of health regard it as equivalent to, if not better than, the old methods. Most of the milk sold as pasteurized is treated in bulk and bottled afterwards. Proper agitation during the heating prevents the formation of a surface film, the layer of coagulated milk protects the bacteria against heat. If the milk is pasteurized in bottles, the bottles should be placed in a covered vessel where the steam will prevent the formation of a scum on the surface of the milk (Fig. 163). The bottom of the bottle should be protected from the vessel by a folded towel or thick cloth so that the flame will not come into contact with the bottom on which the bottle is resting. An exposure of the bottle to a temperature of 150°C. for 20 minutes in such a vessel should be sufficient to kill all the bacteria that pasteurization will destroy, or the bottles may be immersed in water, the temperature of which is brought to 145°F. for 30 minutes, after which it is to be quickly cooled. Many dirt bacteria are spore formers and are not destroyed by pasteurization. In addition to these organisms another group of bacteria, comprising representatives of a number of species of the thermoduric types, thrive at the temperatures at which most organisms are killed or their growth prevented. Pasteurization changes percentage relations of bacterial species in the milk and, while it destroys the majority of the lactic-acid producers, the ordinary pasteurizing temperatures leave them as the predominant group, the number of spore formers remaining about constant. Pasteurization reduces the number of bacteria to a point where the danger from disease is almost negligible, but affects the creaming capacity of the milk, although this makes very little difference if it is to be used as whole milk.

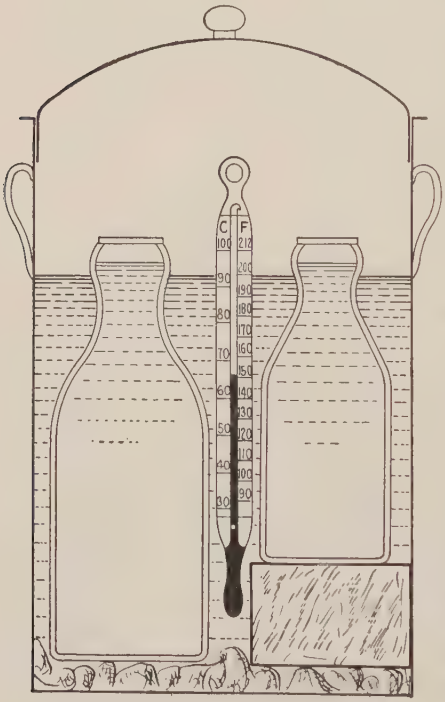

FIG. 163.—Apparatus for home pasteurization of milk in bottles.

MILK STANDARDS

The number of bacteria in milk depends on the conditions under which the milk has been produced, the length of time it has been kept, and the temperatures at which it has been kept. Many cities, to protect their citizens from dirty or old milk, have established certain standards for unpasteurized milk sold within their city limits. The standard for New York City is 1,000,000 per cubic centimeter; Boston, 500,000; Rochester, 100,000; Chicago, 1,000,000 from October to April inclusive. The regulations are purely arbitrary and represent the lowest numbers which it is believed can be obtained and still keep the milk at a reasonable price. An especially high-grade milk can be produced which has lower counts, but such a milk is beyond the means of many consumers.

Score cards for dairies and milk plants have been introduced which give a numerical rating to various forms of equipments and methods employed. A false security is often based upon the results obtained by the use of such dairy score cards.[7] Frequently, under poor conditions and with meager equipment, a careful dairyman may produce excellent milk, while a careless one often produces poor milk with the best of equipment. The methods employed are more essential than the equipment.

Certified Milk.—Many cities are supplied with an especially high-grade milk which is produced under special conditions regulated by a certified medical milk commission. The product must meet the requirements of the Certified Milk Producers' Association of America. The cows are kept clean; they are washed before milking; no dust is allowed in the stable at milking time; the milker washes his hands carefully before milking each cow; and the milk is cooled quickly. The cows are inspected regularly for tuberculosis, or udder inflammation. Such milk may be produced with a bacterial count of about 5,000 to 10,000 bacteria per cubic centimeter. The extra care means extra expense and while ordinary milk may retail for 12 cents a quart, such certified milk is sold at the special stations for 30 cents a quart, a price which puts it out of the reach of poorer families.

Guaranteed Milks.—These milks, produced without the elaborate precautions followed for certified milk, are given enough attention to keep the number of bacteria below that of the ordinary product and to have it from healthy cows, without very much additional cost. Although the quality is nearly as good as that of certified milk, it depends upon the reputation of the dealer or producer and not upon the rigid inspection and supervision of a certified medical milk commission.

Condensed milk is processed milk, containing only about 25 per cent of water, 35 per cent of milk solids, and 40 per cent of cane sugar so that, while the development of organisms in it is very difficult, it is not necessarily sterile. On the other hand, *evaporated milk*, containing no cane sugar, must be nearly sterile in order to keep. The latter milk when entirely sterile should keep indefinitely.

Determining the Number of Bacteria in Milk.—The usual method of determining the number of bacteria in milk is to dilute it with sterile water and then add it to a medium which will permit the growth of the bacteria, counts being made after 48 hours' incubation at 37°C. The disadvantages of this method are evident since, after 48 hours have elapsed, the milk has already been consumed. The numbers obtained serve to warn the consumer as to the general quality of the product he is buying, but the particular lot has been already used.

In order to overcome this difficulty which the ordinary plating methods involve, at least three other methods have been proposed: (1) the little-plate method of Frost;[13,14] (2) the direct staining of the sample of milk on a slide as suggested by Breed,[1,2] Breed and Brew[4,5,6]; and (3) the methylene-blue test. Each of these methods has advantages and inaccuracies and none of them is ideal. The best that can be said of them is that they give us some idea of the condition of the milk in a relatively short time, and are useful in rapid laboratory work in city milk control.

The details of these methods are given in the papers cited and it will be necessary only to point out the general procedures used. (1) Frost's little-plate method is similar to the ordinary Petri-dish counts, but the dilutions are mixed with nutrient agar, and a definite-sized spot made on a sterile glass slide. These slides are incubated in a special chamber for 5 to 6 hours, when the plates are dried. If the colonies are of a sufficient size, they are fixed by passing through flame, then into a 10 per cent solution of acetic acid in 95 per cent alcohol; after which they are stained in methylene blue for 3 minutes and decolorized for a few minutes. The colonies may then be counted under the low power of the microscope. (2) The Breed method is not applicable to high-grade milk, but the types of bacteria and their arrangement, as observed under the microscope, can be used to advantage to correct faulty production if the milk is of poor quality. With a special pipette, $^1/_{100}$ cubic centimeter of the sample is drawn up and then spread out over an area of a square centimeter on a glass slide. The milk is allowed to dry; the protein is coagulated by placing the slide in 95 per cent alcohol; dried again; the fat dissolved out by immersion in xylol; stained in a weak solution of methylene blue; and examined under the oil immersion lens. The draw tube of the microscope should be adjusted so that the size of the microscopic field will have a diameter of 0.205 millimeter and one organism indicates a bacterial content of 300,000 per cubic centimeter. In all cases, a number of fields should be counted and the average taken. Clumps of bacteria are counted as single organisms. No distinction can be made, of course, between the bacteria that are dead and those which are still alive. The method has been compared with the plating-out method and found to give a fair picture of the condition of the milk. The advantage of this method is that a sample can be examined in a few minutes. (3) The methylene-blue test is still simpler in operation and requires no apparatus other than some methylene-blue tablets and a tank where the samples may be kept at a warm temperature. The results are derived from the bleaching of the methylene blue by the reducing power of some of the bacteria which are in the milk. One cubic centimeter of the methylene-blue solution is added to 10 cubic centimeters of the milk, and the time required for decolorization is noted. The method seems to give as good a test for the keeping qualities of milk as either agar plates or a direct examination of the sample.[11]

THE SPREAD OF DISEASE BY MILK

Disease organisms, both from the cow herself and from infected persons, may be spread by milk. The most common disease to which the cow is subject, as well as human beings, is *tuberculosis*. The common diseases which milk spreads, but not of bovine origin, are *typhoid fever*, *scarlet fever*, *septic sore throat*, and *dysentery* (Fig. 164).

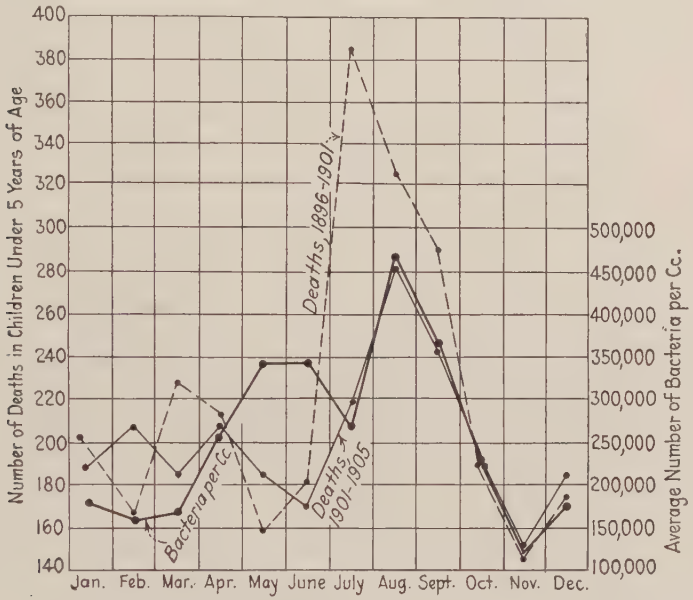

FIG. 164.—Deaths of children under 5 years and the number of bacteria in milk form very similar curves. High counts in milk induce intestinal disorders. (*From Goler.*)

Tuberculosis.—The question of the spread of tuberculosis has already been discussed (p. 285) but it may be well to point out the effects of this very resistant bacterium on the process of pasteurization. The thermal death point of this bacterium is about 60°C. when exposed for 30 minutes, providing the suspension is distilled water, broth, or a saline solution, but milk may hold the bacteria alive, even if the exposure at this temperature is extended to 60 minutes.

Since the real danger of infection in tuberculosis is from the milk which comes from cows where the udder is badly affected with tuberculosis and the organisms pass into the milk, de Man[27] obtained the thermal death point of the organism in this cheesy material, with the following results:

Temperature	Exposure	Temperature	Exposure
55°C. (131°F.)	4 hours	80°C. (176°F.)	5 minutes
60°C. (140°F.)	1 hour	90°C. (194°F.)	2 minutes
65°C. (149°F.)	15 minutes	95°C. (203°F.)	1 minute
70°C. (158°F.)	10 minutes		

The results were often quoted and were made the basis of practical pasteurization measures until the appearance of the work of Rosenau[31] which has become the American standard. Rosenau used milk instead of broth to suspend the organisms while they were being exposed to the various temperatures, the survival of the organisms being determined by their ability to produce lesions after inoculation into guinea pigs. Exposures were made at 60 and 65°C. for varying lengths of time. No survival, as indicated by an infection, was obtained after 1 minute exposure at 65°; infections were secured with cultures exposed at 60° unless the time was lengthened to 15 minutes after which no infection could be seen. As a result of these tests, the recommendation is made to pasteurize milk for 20 minutes at 60°C. The additional precaution must be taken that all the milk is exposed during the entire period and that no scum forms on its surface.[30,24]

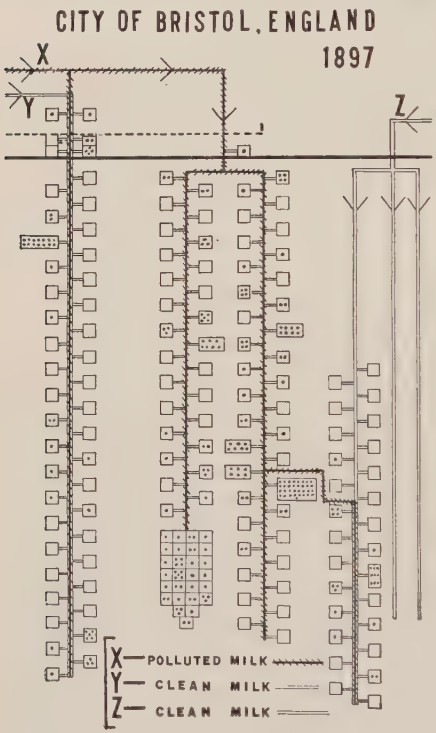

FIG. 165.—An English epidemic of typhoid fever due to an infected milk supply. The infection was from the farm marked *X*; the other dealers *Y* and *Z* bought milk from this dealer and part of their customers became sick. (*From Swithinbank and Newman.*)

Typhoid Fever.—Many serious although small epidemics of typhoid fever are the result of the introduction of typhoid organisms into milk intended for human consumption. Typhoid bacteria come exclusively from human sources, since cattle are not subject to this disease, often from a typhoid-fever carrier who milks or who, in some manner, comes in contact with the milk. Cases of typhoid fever in isolated farmhouses may be traced to the person who does the milking, the pathogenic bacteria being introduced from the hands into the milk. Typhoid outbreaks due to milk are small, as a rule, since they occur only along a milkman's route.

One of the most interesting and complicated of these epidemics[35] (Fig. 165), occurred at Clifton, one of the suburbs of Bristol, England, in 1897. This town was supplied by three milk dealers, X, Y, and Z. Milk from Y and Z farms was uncontaminated, but some of the customers of these dealers came down also with typhoid fever, although more cases were along the route of dealer X. This led to an investigation of the sources of the milk of each man, when it was found that, at certain times, if milk was a little short on the Y farm, this dealer would meet X dealer outside the city and buy a supplementary supply. It was found, further, that dealer Z often did not have milk enough to finish out his route, and that he would get supplementary supplies from the same source. The portion of the Z route supplied with the uncontaminated milk had no cases of typhoid except one, where the servant had bought milk from a shop supplied with X milk. No contamination seemed to have occurred on

farms Z or Y, so the inquiry centered on farm X. The water used for the washing of the dairy utensils came from an open well which communicated directly with a brook which ran very close to it, being really an open sewer draining a nearby village. A farm laborer living beside this brook had typhoid fever some time before, and his excreta may have found their way into the brook and contaminated it. He probably contaminated the brook, the water from the brook percolated into the well, and the well water was used to wash the milk containers.

The first investigation in an outbreak of typhoid is the milk supply of all houses where cases occur. If they all receive milk from the same dealer, the suspicion at once falls on him as the source of the infection. An epidemic due to water would affect the entire population and the cases would be scattered indiscriminately, while a very small outbreak in a single family would be due, in all probability, to some member of the family returning with the disease from a journey to some other city, to a servant who was a carrier, or to a visit from a carrier. Typhoid fever is one of the most interesting of diseases in which to study *epidemiology*.

Scarlet Fever.—This disease is easily spread by milk since many very mild cases are difficult of diagnosis or are not likely to be called to the attention of health officials, and many recognized cases are not well isolated nor a strict quarantine maintained. In either case, the ultramicroscopic organisms may get into the milk from human beings coming in contact with it, either on the farms on which it is produced or by the dealers who handle it.

Septic Sore Throat.—Septic sore throat, due to an infection of the throat streptococci, has often been traced to a milk supply. The milkers may have such an infectious throat and, not considering it to be of sufficient importance to stop work, continue to infect the milk or may return to work with the throat still infected with pathogenic streptococci. These organisms, like the typhoid germ, find in the milk not only a means to survive, but, probably, also to increase in numbers since milk is a perfect food. Staphylococci are associated with ordinary colds and sore throats, but the streptococcic type of infection leads to a swelling of the tonsils, a pronounced inflammation of the mucous membrane of the pharynx, and a general weakened condition of the patient.

Diphtheria.—Diphtheria is spread, in many cases, by the use of milk which has been handled by employees who were suffering themselves from the disease or from cows having an inflammatory condition of the teats or udders.[29]

Diarrhea.—Since children under two years of age are largely fed on milk and are very susceptible to intestinal irritations, especially in hot weather, milk, if it contains the organisms which inflame the mucous membranes of the intestines, is much worse for them than it is for adults. The number of deaths in children under five years of age during the year almost follows the number of bacteria in the city milk supply. These deaths are most numerous in the summer months (Fig. 164), at the time

when the bacteria in milk are also most abundant; numbers drop off during the winter in both cases. Pasteurization of the milk and the establishment of city milk stations, where high-grade milk may be obtained at reasonable prices, are used to combat these conditions. The toll of deaths is always heaviest among the poorer classes of the city who are unable or unwilling to buy good milk for the small children; the well-to-do classes get milk in which bacterial numbers are low and the milk produced from cows that have been tested for tuberculosis. This milk may not necessarily be the certified milk previously described but it is high-grade milk. The poorer classes are often ignorant of the dangers which they run in the promiscuous purchase of milk from unknown sources. Good milk, especially for children, means more care in handling and a higher price but is worth it for children's use.

CREAM

The number of bacteria in milk is generally supposed to be less than the number in cream and the cream standards established by many cities allow 300 to 500 per cent more in cream than they do in milk. These differences between milk and cream are due to the action of the fat globules carrying the bacteria upward as they rise to the surface. With the modern centrifugal separator, however, the conditions have changed,[26] and the numbers are no larger in the cream than they are in the whole milk, although a little higher than in the skim milk, as the following figures show:

	Bacteria per cubic centimeter	
	Separator	Gravity
Whole milk	435,240	135,000
Cream	500,830	285,680
Skim milk	312,740	33,556

With a clean separator, the numbers are not materially changed and cream should contain no more bacteria and should keep as well as ordinary milk.

Ice Cream.—Ice cream has become one of the principal dairy products and is subject to the same bacterial changes which characterize cream kept at low temperatures. The number of bacteria in the cream after it is frozen is actually more, as indicated by plate counts,[10] than in cream before it went into the freezer. This average increase of 48 per cent is due to the breaking up of the clumps of bacteria and not to an actual division in the cells present. Much of the ice cream is made and stored for some days or even weeks before it is used, the temperature being kept

low enough to maintain it as a crystalline mass so that its bacteria, held in one position, cannot move out of their excretion products to find food, and thus decrease in almost all cases.[10,15] Fourteen samples stored for 120 days showed a sharp drop in the numbers after the first 15 days of storage, with a gradual diminution until the end of the experiment, over 40 per cent of them surviving.[10]

BACTERIA IN BUTTER MAKING

Butter is made (1) from the cream as it comes up on the milk or from the separator, or (2) from cream which has been allowed to sour. Sweet cream gives butter with very little flavor, which will not keep in an edible condition a very long time, but which is the favorite type of southern Europe. Sour-cream butter is the common type of the north of Europe and of the United States. The cream is allowed to sour from the action of the lactic-acid bacteria that are in the milk, or from the addition of special cultures. The old-fashioned country butter of a generation ago was made by the natural souring of the cream, but modern creameries depend on cultures (starters) of the sour-milk organism, *Streptococcus lactis*. The objection to the older method lies in the uncertainty of uniformity of the product. A creamery, in order to hold its customers, must produce a uniform article regardless of the time at which it is bought. The cream which comes into a creamery is from all sorts of sources; it may be clean or may be full of dirt organisms which may produce off flavors. The most reliable procedure, therefore, is to kill off practically all these bacteria and then introduce a type which will bring about a definite reaction and flavor. This process is known to the butter maker as the "ripening of the cream." The starters which are used are cultures of the lactic-acid organism alone or mixed cultures containing other types supposed to produce flavor. The sour-milk streptococcus produces acid but will not give a flavor to the butter, so that some manufacturers of starters sell a mixed culture in order to give both acid and flavor. The cream is pasteurized and then a culture of the starter is added to it, the resulting product having much more uniformity than butter obtained without inoculation although not having the very fine flavor occasionally found in country butter ripened from bacteria normally present in the cream.[16,17]

The Keeping Properties of Butter.—Sour-cream butter will keep for some time, especially if kept in storage below 0°C., so that June butter is now on sale at all times during the year. The acidity together with the 14 per cent salt which forms a quite concentrated solution in the water included in the butter is sufficient to check the rapid growth of many of the organisms. The rancidity which develops in old butter is due to bacterial action but the particular organisms which cause this chemical change are not known and the portion of the butter attacked is not certain. It must be recalled that the butter still contains traces of many substances in addition to the fats; among them are the caseins and milk sugars, both readily attacked by bacteria with butyric-acid formation.

THE RIPENING OF CHEESE

The ripening of many soft cheeses is due to the action of molds, but hard cheeses are believed to ripen from bacterial activity. The milk which goes into making cheese, usually soured by *Strept. lactis* must not contain too many *B. lactis ærogenes* or the curd will be filled with large holes and the production of acid too small. The early phases of the ripening seem to be dominated by the ordinary sour-milk organism but in the later months a different set of organisms develops.

The ripening process is not the result of the activities of any one set of organisms but of a succession, each living on the products which the previous variety has produced. All investigators seem fairly well agreed that the ordinary sour-milk organism is not a casein digestor and that the ripening process must result from the action of other species.

The ordinary Cheddar cheese is ripened by the *Bacterium casei* group of organisms which are present in the milk and not by the *Streptococcus lactis*. Cheese made from

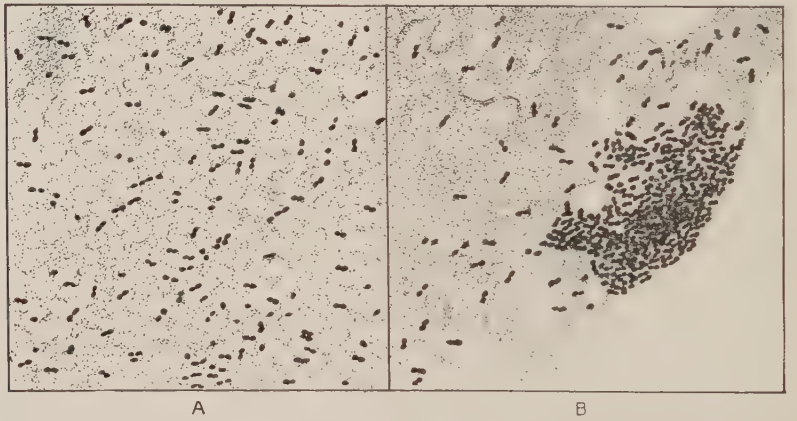

FIG. 166.—The distribution of bacteria in cheese: *A*, 1 month old, bacteria in pairs uniformly distributed; *B*, 6 months old, same or similar organisms in clumps. (*After Hucker.*)

pasteurized milk inoculated with the lactic-acid organism will not ripen.[12] The ripening process is the result of the interaction of several members of the *casei* group, any of which would be unable to complete it but whose combined efforts, one variety using the materials rejected by the others, digest the proteins, change both the physical and chemical properties of the cheese, and give it the characteristic flavor. The flavor is the result of the production, by bacteria, of some of the volatile fatty acids probably arising from citric acid, lactose, or protein. Some of the strains of Streptococci may be responsible for the flavors as they have been found to produce large amounts of alcohol and esters.[34,18]

Hucker,[21,22,23] in his studies of the various types of organisms found in ripening cheese, has made use of sections to determine the bacteria at its various periods, bits of cheese being imbedded in paraffin and sectioned on a microtome. The sections were stained according to the Gram method, and then with methylene blue. The bacteria in the young cheeses consist largely of *Streptococcus lactis* distributed uni-

formly throughout the curd (Fig. 166), but after the cheese has ripened for 6 months, the organisms are mostly in clumps consisting of cocci and a few rods and the cheese seems to be ripened in the region around the clumps. The significance of the clumps is not clear, but certain types of organisms survive to form them.

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CHAPTER XXXVI

MICROORGANISMS IN FOODS AND CANNED GOODS

Microorganisms, abundant in uncooked food substances, are derived from dust particles, from the soil, and from the animals and plants which produce the food. Many of these organisms bring about changes in the food, some of which are beneficial but most of which are detrimental. In either case we try to control the changes which they produce in order to obtain a product which meets human wants. Some of these changes already have been discussed under milk and its products, and the formation of acetic acid as a sequel to alcoholic fermentation.

The spoilage of food by bacterial action takes place in accordance with the material which composes the food. The various groups with the products which are the result of bacterial activity on them may be classed[49] as follows:

1. Proteins produce acids and carbohydrates in traces.
   Example: meat, fish, and eggs.
2. Carbohydrates produce acids and proteins in traces.
   Example: starch, sugar, honey.
3. Proteins + carbohydrates produce acids in traces.
   Example: milk, cereals, flour, greens (cabbage) vegetables.
4. Acids produce proteins and carbohydrates in traces.
   Example: vinegar.
5. Acid + proteins produce carbohydrates in traces.
   Example: hard cheese, sauerkraut.
6. Acid + carbohydrates produce protein in traces.
   Example: must.
7. Acid + protein + carbohydrates produce acids, etc.
   Example: sour milk, butter, fruit.
8. Acids + carbohydrates + proteins in traces.
   Example: water.

Any classification of the reactions fails to cover many individual cases of bacterial activity; about the only method of showing the relation of bacteria to food is to take each food as an example.

The Bacteria in Eggs.—Both white and yolk of eggs often contain bacteria; some of them are included in the egg as it is laid, others penetrate the eggshell and membranes. The ovaries of the hen are often not sterile, as is indicated by the very much higher bacterial infection of the yolk than of the white;[26] others[51] deny general infection of the oviduct in the ordinary healthy fowl. The percentage of infected, freshly-laid eggs varies from 9.5 per cent[51] to 23.7 per cent;[9] the organisms present are staphylococci, spore-forming rods, *B. coli*, *B. proteus*, etc., but no markedly pathogenic

organisms. They are, however, frequently of the type which destroys the egg proteins.

In addition to the bacteria enclosed in the egg at the time it is laid, others find their way into the white through the very porous shell of the egg, and through the fibrous membranes (Fig. 167) which permit the entrance of bacteria and the growth inward of mold filaments. Dirty eggs, especially dirty eggs which have been washed, are more likely to be subject to contamination from the exterior, since washing removes the layer which stops the pores in the shell.[52]

The preservation of eggs is dependent on the prevention of the growth of the organisms already inside the egg, whether derived from the hen or those which have penetrated the shell. This is usually accomplished by the use of clean eggs, low temperatures, and preservatives, such as waterglass, which seal the pores of the shell and prevent the growth of the ærobic bacteria, the predominant type.

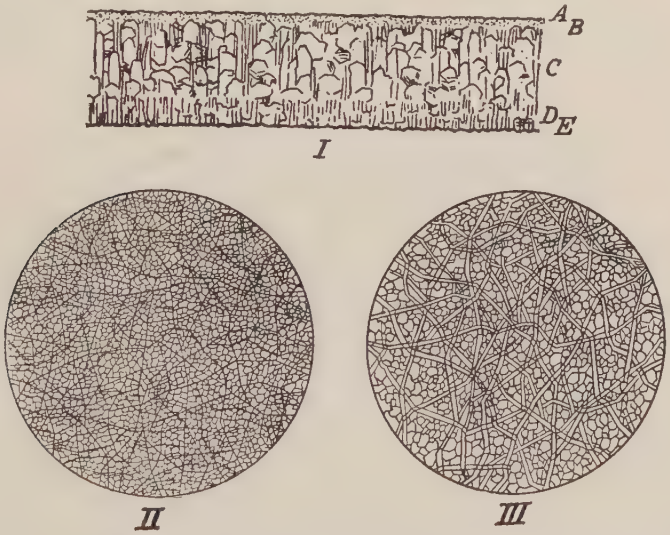

FIG. 167.—I. Cross-section of egg shell; *A*, gelatinous coating of shell; *B*, outer porous layers; *C*, middle granular layer; *D*, inner crystalline portion; *E*, inner face. II. Inner shell membrane. III. Outer shell membrane. The membranes are made of fibers. (*From Benedict.*)

Bacteria in Meat.—The bacteria in meat are introduced from the outside to a large extent and consist of various protein-destroying species, the commonest being *B. proteus*, *B. coli*, *B. ærogenes*, etc. Not all of the bacteria come from the outside; 77 per cent of muscular tissue samples from slaughtered hogs and 84 per cent of the blood of various slaughtered animals show bacteria in cultures.[50]

Meat which has been ground is much more likely to contain numerous bacteria, but if Hamburg steak contains more than 1,000,000 bacteria per gram, it is likely to give external evidence of decomposition. The number varies greatly on different cuts of meat, in general the organisms being mostly of the colon type and putrefactive.[6] The appearance of the meat gives some indication of the number of bacteria which may be expected in it; smoked meats show much less than fresh ones.

The production of hydrogen sulphide marks the spoilage of ground meats,[61] being more positively detected than foul odors. Comparatively good Hamburg steak will give such a test in 7 to 10 hours but will keep in a refrigerator for at least 3 days. If foul odors are present, the hydrogen-sulphide test will show in 2 to 5 hours, but a

slight tainted smell might not mean that the meat had putrefied too much for consumption.

Bacteria in Bread Making.—The yeasts are the important microorganisms in bread making. In the old days when the *leaven* consisted of a part of the dough from the previous baking, the mixed cultures of yeasts and bacteria kept in cultivation gave carbon dioxide and flavor; the very high flavor of the black bread, made from unbolted rye flour in Central Europe, being the result of the lactic, acetic, and, probably, butyric acids formed by the wild yeasts and bacteria in the leaven.

At times, infection of bread trays and utensils with spore-bearing organisms related to the potato bacillus, *B. vulgatus*, produces an epidemic of ropy or slimy dough. The organism, probably introduced in the flour, finding moisture and food in the dough, develops rapidly, especially in summer. It can be prevented by the use of low temperature for the raising of the bread or by the addition of half sour milk and half

FIG. 168.—*Lactobacillus leichmanni*, lactic-acid-forming organism isolated from cereals. Cells filled with metachromatic granules. Stained with dilute fuchsin and decolorized, × 1,200. (*Fred, Peterson, and Stiles.*)

water, instead of water alone, the lactic acid checking the growth of the slime-producing organism.[24]

Salt-rising Bread.—The yeast plant is not the only organism which may be used to raise bread. The so-called "salt-rising bread" much liked in certain parts of the United States is a very fine-grained type with small holes. The organism which raises this bread is a spore-forming bacillus which produces a gas composed[32,33] of two-thirds hydrogen and one-third carbon dioxide, but without the formation of alcohol. The latter feature especially recommends it, as the alcohol produced in bread is an entire economic loss; 3 to 4 per cent of the food value of the bread disappears in this way while in lightening with the bacillus, only about 1 per cent.

Bacteria in Cereal Foods.—Bacteria will cause, in a few days in hot weather, the spoilage of cooked cereals by the formation of acid. German wheat flour contains 14 to 23,000 bacteria in a gram; Russian flour 256 to 309,000, while rye and oat flour or

cereals have even higher numbers. Some of these organisms, spore formers, and acid producers, escape the cooking given to cereal food. Cereals which become damp in the package are very likely also to develop similar defects in flavor from acid production.

One of the usual types found in America on cereals are granule-containing rods, *Lactobacillus leichmanni*, which ferment glucose, fructose, glucosides, or dextrin.[23] The granules of volutin in the long, slender rods make them very easily identified (Fig. 168).

THE ACETIC-ACID BACTERIA AND THE MANUFACTURE OF VINEGAR

The formation of acetic acid may be useful or may be harmful. The original studies on the formation of acetic acid by Pasteur[46,47] were made to show that alcohol would change under the influence of minute organisms into acetic acid and that the process was a biological rather than a chemical one. We wish at times to make use of the acetic-

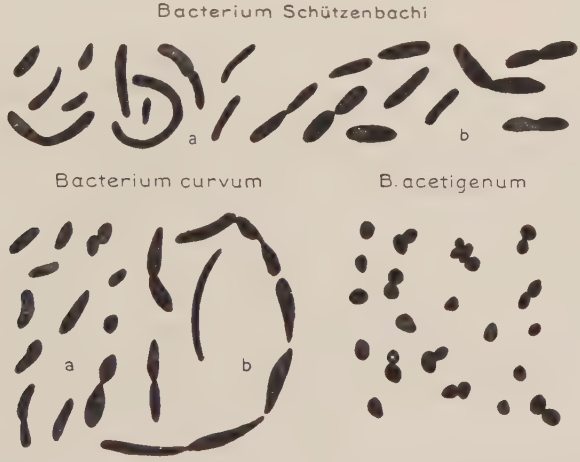

FIG. 169.—Three rapid producers of acetic acid. (*After Fuhrmann.*)

acid forming properties of some of the types which produce it rapidly in quantity. Pasteur's original organisms, supposed to represent a single species, *Mycoderma aceti*, have been split up and new species discovered, so that we know today that at least 15 species are able to produce acetic acid in quantity. A majority of these species have been isolated from wine and beer, but a number of so-called "rapid acetic-acid producers" are known (Fig. 169).

The Organisms.—While the acetic-acid bacteria have been grouped together as a physiological unit largely by investigators interested in the amount and rapidity of acid formation, this arrangement may mean very little, since there are very large differences in morphology. The majority of them are held in the gelatinous film produced by the outer layers of their cell walls swelling and fusing to form a tough continuous mass known as a "zooglea," of which the organisms themselves form only a small portion of the bulk. Some species, recently studied, do not form this typical pellicle.[53]

Three varieties used for the fermentation of alcohol (Fig. 169) show the variation in form characteristic of acetic-acid varieties, but the spontaneous types which appear in cider vinegar, known as "mother of vinegar," are usually rods of various lengths. The usual amount of acetic acid which the latter will tolerate, about 3 to 5 per cent, is sufficient for commercial vinegar manufacture. An attempt has been made in recent years by various agricultural experiment stations (Michigan[21]) to supply pure cultures of acetic-acid organisms which may be added to cider to produce a definite reaction and acidity. The different species vary in their ability to withstand the by-products; *Bact. Schützenbachi* produces a maximum of 10.9 per cent in 66 days or a total of 14 to 15 per cent; *Bact. acetigenum* produces only 3.5 per cent. In addition to acetic acid, esters are always present; these give the flavor to vinegar and form a large part of its value as a condiment.[30]

The formation of acetic acid by alcohol oxidation may not be the only activity of these organisms as, in the depths of the culture solution, they may form directly from glucose, alcohol, carbon dioxide, and gluconic acid; 1 per cent alcohol being found at times.[58]

These organisms grow well in a liquid medium where the entire source of carbon is alcohol, with nitrogen from ammonium salts.[4] Henneberg[27] used for rapid acetic-acid producers, a culture solution made up of the following:

Ammonium phosphate	2 grams
Ammonium sulphate	1 gram
Acid potassium phosphate	1 gram
Magnesium sulphate	1 gram
Distilled water	1,000 cubic centimeters

To this was added 6 per cent dextrose syrup and 3 per cent (by volume) of alcohol as a source of carbon.

The temperature relations for the most rapid fermentation of alcohol have been carefully studied and found to depend upon the organisms used, but to lie between 25 and 35°C.

Since acetic-acid fermentation is essentially an oxidation process, the easy access of the bacteria to an abundant supply has been met by various devices. In culture flasks, growth of the acetic-acid organisms is necessarily slow, since only those in contact with the air have optimum conditions. By allowing the fermenting liquid to trickle over shavings, the latter soon become coated with a slimy growth of bacteria which continue to change the alcohol into acid as it comes in contact with them. The partially converted liquid can then be pumped up and the process repeated. Any source of cheap alcohol can be utilized, so that vinegar is made from cider, poor quality of grape juice, waste honey, cheap syrup, etc.[35]

THE FERMENTATION IN SAUERKRAUT AND SILAGE

The souring of the juices of cabbage gives the food known to the Germans and others as "sauerkraut." In order to obtain a quantity of juice, the cabbage is sliced into thin sections opening the cells so that the liquid portion flows out. The sugar or the protein is broken up into lactic acid The transformation is the result of the activity of the bacteria which are typical lactic-acid formers but it is doubtful whether any one species of organism is entirely responsible. Gruber[25] called the organism, which he isolated from German kraut, *Pseudomonas brassicæ*,

acidæ, but LeFevre[37] claims that the essential organism in American kraut is *Lactobacillus brassicæ* Wehmer.

The lactic acid prevents the growth of putrefying bacteria and as long as mold growth (which will neutralize the acid condition) is prevented, the cabbage will keep. It must be covered at all times with liquid to prevent the access of oxygen.

The souring of silage in a similar manner serves to preserve it from the attacks of the putrefying organisms. This souring[28] is the same bacterial process regardless of the origin of the material composing the silage, the fermentation resulting in the formation of lactic acid by members of the Lactobacillus group assisted in a minor way by other lactic-acid formers. The yeasts are of very little importance except during the very early stages of the fermentation, or as a cause of spoilage when silage is exposed to the air.

THE PRESERVATION OF FOODS

Since bacteria, yeasts, and molds destroy the value and the flavors of foods, if their growth is not restricted in some manner, they may be regarded as our rivals and if they get their work on the foods completed before we can use them, they get and we lose all the benefit. At certain seasons of the years, food is usually very abundant but, during the winter and spring, many summer and fall products cannot be found in a fresh condition and must be preserved. Primitive man soon learned that he could not depend on the products of the field and woods unless he took care to preserve them in a condition somewhat resembling that when fresh, and with their nutritive properties intact. We think little of the wonderful choice of foods which we have at our disposal during the winter months, but if the modern family had to live as did the people of 150 years ago, on a diet during the winter months of bread, dried fish, meat, potatoes, beans, and turnips, varied with a few dried fruits or vegetables, they would think that the pleasure had largely gone from eating. The importance of modern food preservation is so great that it has been classed as one of the nine wonders of the modern world. The preservation methods have, however, in many cases so changed the flavor of the food that we have acquired an entirely new set of gustatory standards.

Drying.—The oldest and simplest of preservation methods was that of *drying*. Dried meat and fruit in a dry atmosphere keep for many months. Drying must be done quickly to prevent spoilage and the slightest moisture is likely to induce the growth of organisms. The addition of various *salts*, especially common salt (NaCl), checks still better the growth of the lower forms of life. Meat that has been salted and then dried keeps much better than meat that has been simply dried; if such dried meat or vegetables become slightly moist, the salt comes to the surface to form a brine in which life is impossible for bacteria or molds. The addition of salt, however, is objectionable in substances, such as fruits, where the large percentage of sugar would give the salted dried product a very objectionable flavor. Boiling fruit in a thick syrup will produce, however, a combination which hardens when cool, in many cases

at least, and this jellied product keeps for some time if yeasts and molds can be prevented from growing on it. Such a spoiling would be largely superficial, however, and jellies and jams could be kept long after the season for fresh fruit.

Drying fruit or meat increases the percentage of salts in the cells. This increase in the osmotic pressure of the nutrient substances in solutions makes it difficult for the microorganisms to absorb anything from them-for growth, and, in fact, if they come into contact with a very concentrated brine, they may be plasmolyzed (Fig. 170).

The plasmolytic effect of the salts in cell sap is further increased in intensity by the addition of salt before the drying process. The salt probably has two effects, plasmolytic and toxic. The chlorine-ion of the salt has a deterrent effect on the growth of bacteria. Since this method of preservation has been used particularly for meats, investigations have been made of the amount that is necessary in order to check the putrefying and ptomaine producing organisms.

The suggestion of Stadler, quoted by Karaffa-Korbutt,[31] that the percentage of salt in the interior of the meat is not of so much importance in the prevention of the

FIG. 170.—Plasmolyzed bacteria: *A*, typhoid bacillus in 2.5 NaCl solution; *B*, cholera vibrios in 0.75 KCl solution; *C*, Spirillum undula, plasmolyzed in drying out, × 500. (*After Fischer.*)

growth of organisms from the outside is probably correct. The concentration on the interior, however, must be sufficiently high to prevent the growth and formation of toxins by the so-called "ptomaine-anærobic bacteria." Karaffa-Korbutt agrees with Tomczak that salt has a weak antiseptic action, particularly against the pathogenic types, 8 to 9 per cent of NaCl being sufficient to check the growth of members of the *B. coli* group to which *B. enteriditis* belongs, although 10 to 12 per cent is necessary to check some of the septic varieties, and wild Tortula yeasts can live in a concentration of 25 per cent. The non-spore bearing types of bacteria die off in a few months in a concentrated solution of NaCl but, of course, the spore-bearing varieties are not affected.

The salt must be applied very freely if it is to stop the growth of the ptomaine-producing organisms which appear in meat. Serkowski and Tomczak[53] found that, in cultures, a 5 to 10 per cent solution would not check the growth of organisms such as *B. enteriditis*, *Proteus vulgaris*, or *B. botulinus*. A 15 to 20 per cent solution would check their growth so that at least a 15 per cent salt solution must be added. If this amount is to be added to fresh meat, however, it is only necessary to give the outer portion of the meat a thorough coating to raise the percentage very high, since this is the place where contamination is likely to occur.

The following table by Thom[56] shows the effect on the growth of some Penicillium species when salt is added to Czapek's culture solution:

EFFECT OF SALT ON THE GROWTH OF MOLDS: THE NORMAL COLONY BEING REPRESENTED AS 1.0.

	Check		With 5 per cent salt		With 10 per cent salt		With 15 per cent salt	
	7 days	34 days	7 days	34 days	7 days	34 days	7 days	34 days
P. camemberti..	0.8	1.0	0.7	1.0	0.3	0.6	0.2	0.8
P. italicum.....	0.8	1.0	0.3	...	0.0	0.4	0.0	0.1
P. expansum ...	0.9	1.0	0.8	1.0	0.5	0.8	0.1	0.4
P. roqueforti ...	0.6	1.0	0.4	0.7	0.3	0.6	0.1	...
P. commune....	0.8	1.0	0.7	1.0	0.1	0.7	0.1	0.8

Effect of Salt on the Bacteria.—The effect of the salt solution is to produce degeneration (involution) forms in bacteria if added in sufficient quantity. Matzuchita[43] found that, while some organisms could stand a culture medium with 10 per cent of salt, with many others the addition of 2.50 to 3.5 per cent of sodium chloride would produce striking degeneration forms. Some coccus species under the influence of salt would change into rods, some of the rods would produce spindle-shaped types, and others develop into spheres. The changes induced by salt in the pest bacillus are so constant that this investigator has suggested that they could be used as a means of identification for this organism.

Salt-resistant Organisms.—While the majority of the bacteria are so susceptible to a high content that they will not grow, although they may not be killed, certain organisms or strains of organisms will survive a brine containing 10 per cent or more of salt and are known as "halophilic" or "haloduric".[36] Such organisms isolated from pickle scum where the brine contained 10 per cent salt will grow on an agar containing cucumber juice to which 10 per cent of salt has been added. Browne[7] isolated two organisms, from spoiled salted fish, able to stand 16 to 18 per cent of salt in the media. These organisms must have a very high internal osmotic pressure to be able to obtain food from a medium containing this amount of plasmolyzing substance.

The Addition of Sugar.—The addition of sugar has the same physiological effect on osmosis in the bacteria as has salt. The amount of sugar usually added to fruit juice for making jellies and jams is the same as the weight of the fruit juice but, as the combination is usually boiled for some time after the sugar is dissolved, the finished product contains this percentage of sugar plus the concentrated fruit juice (with its 10 to 20 per cent) making at least a 65 to 70 per cent solution. Molds will grow on such concentrations so that any food dependent on sugar for its preservation must be kept away from the air, oxygen being essential to mold growth.

Succrodric Yeasts.—Strains of yeast have been found in chocolate-coated creams burst from the formation of gas inside them; the yeast plant grows in the absence of air in the very concentrated sugar solution in the cream.[11] Similar yeasts are found, at times, in maple and other syrups.

Smoking Meat and Fish.—The only chemical which preserves and is not markedly injurious to human beings is developed in the process of *smoking*, pyroligneous acid. Meat hung in the neighborhood of smoldering fire absorbs the gaseous acids from the wood and these act as preservatives. The discovery of this property of pyroligneous acid was probably made by accident in some primeval hut where the venison was hung to the rafters to dry, but it is utilized in the modern way by simply painting the meat with it.

The Use of Vinegar and Spices.—Vinegar and spices are among the old preservatives of foods that are to be kept temporarily, such as catsup, salad dressing, mince meat, and pickles. These foods contain much liquid and present a possible field for yeasts and bacteria.

The growth of bacteria can be readily prevented in catsup by a combination of 10 per cent sugar, 3.5 per cent salt, and 0.4 per cent of acetic acid, but yeasts will grow even in a combination of 25 per cent sugar, 4 per cent salt, and 0.75 per cent of acetic acid.[48] Salad dressing may contain from 0 to 17,000 spore-forming bacteria per gram in spite of the vinegar. The organisms are both ærobes and anærobes but are not, apparently, pathogenic.[2]

Spices are popularly supposed to retard the growth of bacteria, especially cloves, cinnamon, allspice, mustard, pepper, and ginger, but their effect has been overestimated. Unless 2½ per cent of allspice and cloves is added to mince meat, the retardation is not noticeable on anærobic bacteria, such as *B. botulinus*, and such a food is always a possible source of danger unless heated thoroughly.[1]

The Use of Antiseptics and Preservatives.—Chemicals may check the growth of organisms without killing them. So many varieties of this type of preservatives have been made by the chemists that it would seem that some of them could be used for that purpose without affecting the digestibility of the food or the health of the consumer. Unfortunately, any chemical which stops germs from growing is also, to some extent, a poison to the person who eats the food. Some food preservatives that have been used are sodium benzoate, salicylic acid, benzoic acid, and boric acid, or combinations of these chemicals.

The addition of $\frac{1}{10}$ per cent of sodium benzoate to perishable foodstuffs will not prevent their deterioration indefinitely but checks the growth of organisms, such as the wild yeasts which may fall into the catsup, jam, or jelly after it has been opened and before it is completely consumed. These foods are sterile when they are placed in the containers but are contaminated from the air after opening. The small percentage of sodium benzoate added is supposed to have a hindering effect on the growth of the yeasts and molds and not to be injurious to the consumer of these foods. The various yeasts do not react in the same degree to this mild antiseptic or preservative. Tanner and Strauch[54] found one strain of *S. ellipsoideus* completely inhibited in a solution of 0.15 per cent but others showed growth with 0.4 per cent and some grew uninjured even in a 1 per cent solution. The 0.1 to 1 per cent usually added would seem, therefore, to be of use only in somewhat retarding growth. The use of the so-called "canning powders," usually boric acid, will not prevent the growth of spoilage bacteria in canned goods when used in the amounts recommended[17] and are undoubtedly injurious to the health of the consumer.

The use of preservatives, such as sodium benzoate, even in a very weak solution (one-tenth of 1 per cent) has been questioned by health officials and many manufacturers of food products no longer use them. Such substances are added to jams, jellies and catsups, which are opened some time before they are entirely consumed and which frequently may be recontaminated, the claim being made that the preservative controls the organisms which the consumer has introduced and is not to conceal the faults or unsanitary methods of the manufacturer.

FOOD PRESERVATION BY CANNING

The methods of drying, salting, smoking, and preserving with sugar were known to our forefathers before 1800 and were in common use for the preservation of food for the winter months.

The Discovery of Canning.—All of these methods, however, were inferior to the method first introduced by Appert in France in 1810, by which food of any sort could be cooked and placed in containers and would keep as long as they were unopened or uninjured; in this method, the air must be kept away from the canned contents. For his discovery, Appert received a prize of 12,000 francs from the French government. The surprising part of this discovery is that it was made long before much was known as to the importance of the bacteria, yeasts, and molds in

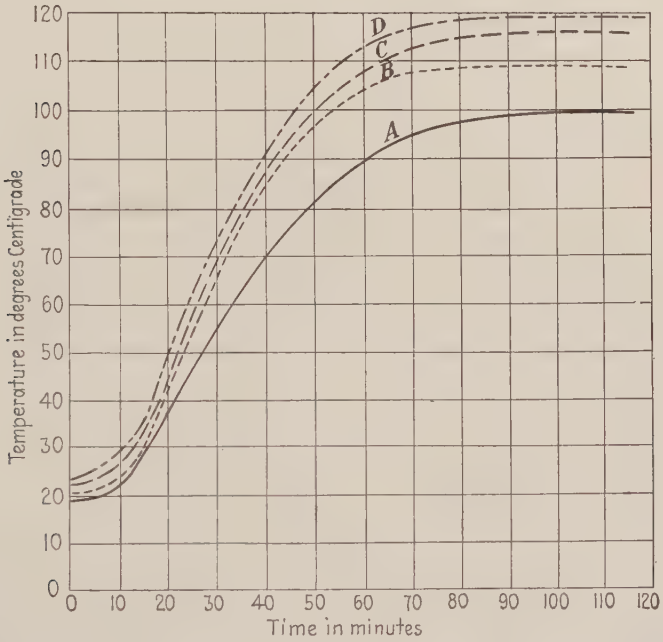

FIG. 171.—Rise in temperature in center of pint glass jar of sweet corn processed at *A*, 100°C.; *B*, 109°C.; *C*, 116°C.; *D*, 121°C. (*After Magoon and Culpepper.*)

the spoiling of food and is a good example of the way in which practical men stumble on important discoveries, although they may know nothing of the fundamental principles involved.

Home and Commercial Canning.—Canning has become such an important feature of modern life that we should feel a great loss if unable to get the numerous canned varieties of foods. A large part of these are put up in the home but, in many farming regions at the present time, a large portion of the product goes into canning factories run only in the season when there is material to can. The bacteriological problems are basically the same in both cases but they are met somewhat differently, owing to the limited equipment in the home. In the factory the

heated material is usually placed in the cans after being partially cooked, the cans sealed and sterilized. In the home, the material is partially cooked, placed in the glass jars or cans cold, the containers partially closed, and sterilized, the containers being sealed after they are taken from the sterilizer. In the factory, the sterilization is produced by means of steam under pressure, the pressure giving a temperature between 240 to 250°F. which is maintained for a few minutes for fruits and tomatoes, and for over an hour for peas, corn, beans, and other vegetables.

Tomatoes and Acid Fruits.—The bacteriological problems involved in these canning operations are numerous, depending on the material to be canned and the method used. With tomatoes or fruits the problems are much simplified, since the acid juice checks the growth of bacteria and only yeasts and molds have to be killed. A few minutes' boiling, or steam under pressure for 5 minutes, will destroy all living things that will grow in canned tomatoes or fruit.

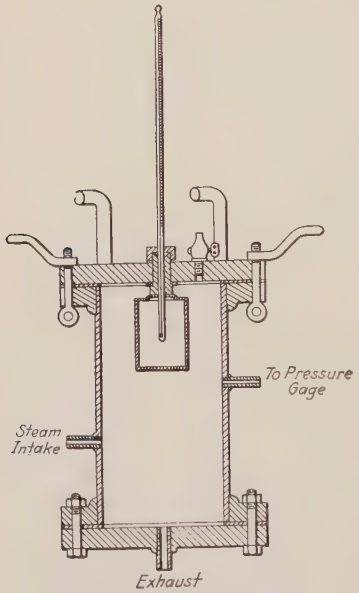

FIG. 172.—Apparatus used for determining temperature in jar or can surrounded by varying pressures. (*Magoon and Culpepper.*)

Peas, Beans, Corn, Meat, and Chicken.—The problem becomes more complex with the canning of meat, or of vegetables which are not acid; the canned material must be rendered practically sterile. This involves killing not only the vegetative bacteria but also the spores. The former are easily killed by a simple boiling but the spores are more resistant and higher temperatures and a longer time are essential.[16, 62]

The bacteria which are to be found in peas, beans, corn, etc., are of the soil types and the spores are among the most resistant known. Washing with water and blanching frees the material from much of the dirt which adheres to the pods or the grains but the chief dependence must be on the steaming process after the material is placed in the cans. The method followed is the *hot pack* where the material is put, as hot as possible, in cans which are then sealed, and placed in a retort where the temperature of 240 to 250°F. is maintained for 40 to 60 minutes. Both cooking and sterilization are thus done almost entirely in the can itself and the heat must penetrate to the center of the can to maintain a temperature at which the spores will be killed. The penetration of the heat into a glass jar of sweet corn, shown in Fig. 171, is characteristic of other readings obtained.[39, 40] The jar is attached to the lid of the retort by a screw and through the screw a thermometer issues to the exterior (Fig. 172); the temperature at any time may be read on this thermometer.

In commercial canning where heated material is placed in ordinary tin cans, it exerts an influence on the temperature induced inside the can when processed at a high temperature. The temperature in the interior of the cans which have been filled with hot material rises more quickly than in cans filled cold. The cubic contents of the can change under the influence of the expansion of the water in the canned liquid. The following table[40] shows the expansion of the ordinary No. 2 and No. 3 cans under different pressures:

Pressure per Square Inch in Can	Increase in Volume in Cubic-centimeters	
	No. 2 Cans	No. 3 Cans
2½ pounds	5.4	28.3
5 pounds	12.1	37.1
10 pounds	18.0	46.0
15 pounds	22.6	54.6

The initial volume of water in a No. 2 can is approximately 550 cubic centimeters and of a No. 3 can 950 cubic centimeters so that the increase amounts to about 5 per cent of the volume. The graph in Fig. 173 shows the rise in temperatures if string beans are placed in cans at various temperatures. In 8 minutes, the interior of the can is the same temperature regardless of the initial temperature of the beans when they were placed in the cans, but the processing time is shortened just that much, so

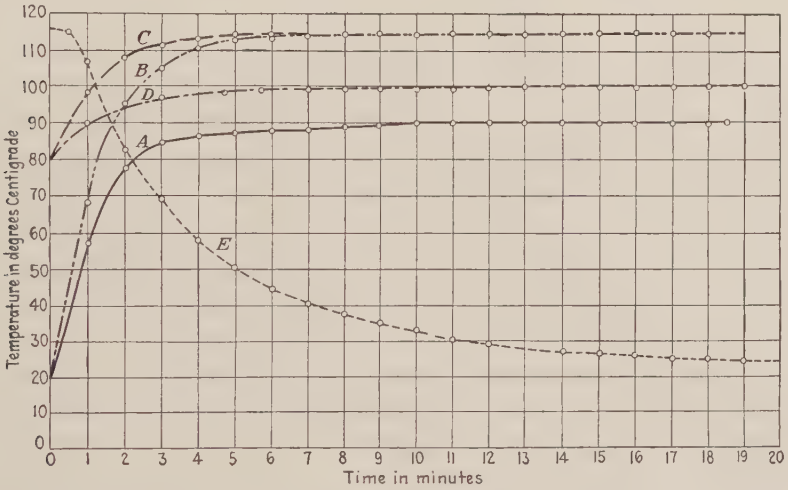

FIG. 173.—Temperature in No. 3 cans of string beans filled at different temperatures and processed at different temperatures The cooling of a can when placed in water at 20°C. *A*, sealed at 26°C. and sterilized at 116°C.; *B*, sealed at 26°C. and sterilized at 116°; *C*, sealed at 80°C. and sterilized at 116°C; *D*, sealed at 80°C., and sterilized at 100°C.; *E*, cooling curve of a can starting at 116°C., cooled in water at 20°C. (*After Magoon and Culpepper.*)

that allowance must be made where the cold-pack method is used in order to allow additional time for the heat to penetrate. The cans must be made strong enough to allow them to spring out at the ends from this internal pressure.

Home Canning.—In home canning, the cold-pack method is usually followed and sterilization is effected either by heating the cans or jars for 3 hours in a steamer or autoclave at 5 to 10 pounds pressure for about 1 hour. Three hours' steaming will gradually destroy the spores, perhaps by the imbibition of heated water into their interior, while steam under pressure will effect the same result in a shorter time.

Three-day steaming seems to have fallen into disfavor and it is not used now to any extent for canning operations, a three-hour steaming on the same day seeming to produce the same results. The principles involve the destruction of the spores of soil organisms such as *B. mycoides*, or members of the *B. subtilis* group, present at all times on fresh corn before sterilization. Some of them are very heat-resistant,

although none of them will withstand the temperatures used in commercial canning.[29] But, as has been already shown, the older idea that spore germination was a lengthy process involving many hours is probably incorrect. The observations already quoted (Fischoeder[22]) on the spores of the anthrax bacillus, that even a few minutes' exposure to favorable conditions, such as an abundance of food, would induce internal conditions which would almost entirely destroy the resistance of the spores to disinfectants and to steaming, may apply also to those found on peas, beans, corn, and meat.

Less heat is necessary to kill bacterial spores in an acid solution of fruits or vegetables than in a more nearly neutral one, or the same heat can be applied a shorter time. At a temperature of 120°C., the time necessary to destroy spores of some thermophilic organisms in a medium of pH 4.6 was about one-fourth the time required if the medium had a pH 6.0.[5] On the other hand, the salt which is usually added somewhat protects the spores against the action of the heat.[59]

Forms of Spoilage.—A common form of spoilage is the conversion of the sugars or the proteins into lactic or other acids with the formation of gas. This spoilage, of course, renders food unfit for human consumption and although it may not be actually dangerous to health, makes it distasteful and unpalatable.

Understerilization of canned goods results in: (1) "flat sours," (2) "swells or flat sours."

From spoiled (flat-sour) non-acid foods, cultures of thermophilic spore-bearing facultative anærobes, and from swells, obligate anærobes, may be isolated. In spoiled acid foods, the organisms may be either bacteria or yeasts; some of the yeasts form gas, others do not.[20]

The flat-sour organisms may be divided into two groups: facultative thermophiles and obligate thermophiles. The organisms which cause flat sour in canned corn are large, ærobic (facultative anærobic) bacteria. They are spore-forming thermophiles growing best and most rapidly at about 60°C., but continuing to grow at as high as 76°C. and as low as 45°C. The spores are so resistant that the organisms may grow in canned goods and cause the acid fermentation even after the ordinary processing. They are found not only in canned corn but also in peas, string beans, pumpkin, and tomatoes.[14,10]

POISONING FROM EATING CANNED FOOD

The use of canned goods which have spoiled is not always attended by any danger to the health of the consumer, but canned food containing a high percentage of protein, such as beans, peas, meat, or fish, is very often dangerous. The destruction of proteins under the anærobic conditions which occur in the interior of cans or jars produces split proteins known as "*ptomaines*" which are very highly toxic when taken into the human intestine. These bodies have a complex chemical composition; *cadaverin*, the first one isolated, for example, having the formula $NH_2C_5H_{10}NH_2$. Some of them may be split further very easily into simpler compounds which are harmless. The original ptomaine poisoning, such as that described by Liebig[38] as occurring in the interior of sausage in Wurtemberg about 1839, has been found to be due to the action of the organism, *B. enteriditis*, which makes its way into the meat from external contamination from the fecal matter in the intestine of animals. The poison produced by this organism is not,

however, a true ptomaine but is really an endotoxin held in the body of the bacillus. It is very resistant to heat and cannot be destroyed by boiling, as can many so-called 'ptomaines" now more properly called "toxins"; the word "ptomaine" should probably be dropped entirely and "toxin" substituted.

Another food-poisoning organism is *B. botulinus*, introduced into food from soil where it occurs in abundance.[44] One type of it has been found in about 20 per cent of the soils and composts from European countries and in about 10 per cent of the various fruits, vegetables, and feeds examined from this country. This organism is an anærobe and a spore former. The outbreak of food poisoning in various parts of the United States between 1919 to 1924 resulting from the eating of canned ripe olives which had been imperfectly sterilized[12,57] caused the National Canners' Association to make possible a thorough study of the botulism organism during the past 6 or 7 years. The ripe olives causing this trouble had been imperfectly sterilized, the spores being still virile inside the cans. The anærobic conditions being no deterrent to their growth, they produced *a substance highly toxic but readily destroyed by heating.* As canned ripe olives are never heated but are eaten as they come from the can, the poison was ingested undamaged. The same organism probably often occurs in other canned foodstuffs, such as spinach,[34] but since a majority of canned foods are heated before being eaten, the toxin is rendered harmless.

The death rate in botulism is very high[13]: of 64 cases in the United States between 1894 and 1918, 41 (or 64 per cent) died, the percentage in the outbreaks varying from 28 to 100 per cent.

In order to eliminate the danger of such poisoning, Thom[56] makes the following suggestions:

A. Clean, fresh, sound food will not cause botulism.

B. Food freshly heated to the boiling point will not cause botulism.

C. Recooking is advised whenever opportunity for microbial activity has been given, but only before spoilage becomes evident by appearance, odor, or tastes. Food showing signs of decomposition is potentially dangerous, hence physical evidence of spoilage should lead to destruction, not to salvaging.

D. Intelligent cooperation of packer, dealer, and householder in the examination of food before it reaches the table is essential to the prevention of botulism.

The Two Types of Poisoning.—The poison from *B. enteriditis* is fast in its action and usually causes death in from 1 to 2 days after the foods are taken into the alimentary system. Digestive disturbances may be caused by members of the Proteus group of bacteria but the symptoms here usually noted are violent vomiting and diarrhea. The *B. botulinus* toxin produces its effects from 8 to 120 hours after being swallowed. The effects are a paralysis of the muscles of the eyes to produce double vision; nausea accompanied by vomiting; accumulation of mucus in the throat, difficult swallowing accompanied by choking; and broncho-pneumonia.

The Organism.—*Bacillus botulinus* is a rod, 0.9 to 1.2 microns in width and 4 to 6 microns in length, usually found single or in pairs; motile by 4 to 8 flagella; a spore former, the oval spores being produced usually at the ends of the organism. It is a strict anærobe but will grow under slightly ærobic conditions if ærobic organisms are present to utilize the oxygen.

Three strains of the organism are known, types *A*, *B*, and *C*, the differences between the three strains being largely physiological.

The botulism organism has not been isolated from a large percentage of the materials which have been supposed to have caused outbreaks; only about 19 per cent of the German cases have been shown to be due to bacteria and 34.6 per cent of the American, the difficulty lying in the isolation and demonstration of the organism. Dubovsky and Meyer[15] grew it very successfully under anærobic conditions on a beef-pepsin-liver broth where it produced toxin in abundance, if incubated at a temperature of about 35°C. for 10 to 12 days. At the end of this time, if the strain is a marked toxin producer, 0.00002 cubic centimeter to 0.0001 cubic centimeter of the broth is sufficient to kill an average-sized guinea pig, but if the cultures are kept longer, the toxin loses in strength. The method which seems to be most successful for determining the presence of the organism in various substances is growth of isolated anærobes in culture media with subsequent inoculation into guinea pigs.

The botulism organism will also grow under anærobic conditions on liver, brain, serum, olive, corn, meat mash, peptone, milk, and digest-media, although the bean and olive infusions will not give a growth that is sufficiently strong to produce typical biological reactions. On 1 per cent glucose-peptone medium, the organism produces an abundance of gas and an abundance of acid which finally stops its growth.[60]

The Toxin.—The organism produces a toxic substance, but whether as an excretion from the body of the bacterium, or as intermediate, poisonous compounds formed by the organism growing from the medium, is not clear. If the latter, it would more properly be called a ptomaine according to the older viewpoint but later workers seem to have entirely abandoned the ptomaine conception. Dickson[13] considered it a true toxin, not only in food substances but also in cultures, since it can be neutralized by an antitoxin, if inoculated into a guinea pig, the effect being neutralized by a simultaneous injection of the antitoxin.

Some authors refer to it as an enzyme, the relationship of toxins and enzymes not being clear. Some investigators believe now that toxins are only special types of enzymes.[41,42] The toxic substance produced by this organism is either an intermediary protein-decomposition product or is intimately connected with such a product which is either with difficulty, or, not at all, diffusible through membranes. It seems to be associated in some way with the proteoclastic enzymes, inducing one of the stages in the degradation of the proteins. When this degradation is complete, the toxins disappear. The toxin itself, however, does not seem to be attacked by the proteoclastic ferments, such as pepsin or trypsin, and so would not seem to be a protein cleavage-product, as it cannot be further digested.

The toxin, or enzyme, is not digested in the stomach or intestine but passes unchanged into the blood stream, doing no injury to the tissues until it reaches the central nervous system where it induces visible changes in the ganglion cells. Degeneration begins at the pole of the cell and spreads inwards to the nucleus which seems to shrink in size, the bits of chromatic material scatter throughout the cell and disappear so that the whole cell becomes homogeneous. It assumes highly vacuolate amœboid forms, after the nucleus has finally entirely disappeared. While the cells of the central nervous system are being altered in this manner, the person having ingested the toxin shows symptoms of the disease within 24 to 36 hours after eating, death being very frequent. The toxin seems to have an affinity for the cells of the

central nervous system alone and does all of its injury there, although other symptoms indicate the disease.[45]

Death of the Spores.—The statement by van Ermengen,[18,19] the discoverer of this organism, that the spores are fairly easily killed by heat, being much more susceptible than many from the soil (½ hour at 80°C. or boiling for 5 minutes) is not entirely correct.

The thermal death point varies with the amount of acid in the substance to be preserved.[63] Thus, in canned foods, such as fruits which are acid, the spores may be killed by a temperature of 100°C. for 50 minutes, while in vegetables which are neutral, 90 to 180 minutes at the same temperature would be required. The substance in which the food is preserved, such as syrup, also affects the death rate of spores of *B. botulinus*. Spores produced on different media vary in their susceptibility to heat and some strains resist boiling for 2 hours, putting them with other resistant soil types.[8]

Killed by Air.—The anærobic conditions must be strictly maintained, for an exposure of 10 minutes to air will kill the vegetative bacteria, although the spores survive on agar in air for 8 days, at low temperatures much longer, and have been kept 500 days.[3]

Occurrence of the Organisms.—Meyer and Dubovsky[44] gathered 1,538 specimens of soil, vegetables, refuse from hog pens, fodder, corn stalks, foods, silage, etc., from various portions of the United States, exclusive of California, and developed from them 375 toxic cultures (16.5 per cent) or, including California, 21,162 specimens with 581 toxic cultures (18.2 per cent). The spores of the organism would seem, from the data, to be very widespread in all soils and refuse, although the presence of a few spores might not be sufficient to start an outbreak as they might not produce enough toxin. Two types are widely distributed; the *A* type being especially abundant in the soil of the western and middle western states, while the *B* type is found more in the middle and eastern states. The *A* type being the dangerous one, the outbreaks of botulism are more common in the West than they are in the eastern part of this country, unless infected canned food from the western region is shipped to other regions and eaten.

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CHAPTER XXXVII

PATHOGENIC BACTERIA IN MAN AND THE ANIMALS

Some organisms which produce disease in man and the animals have already been discussed in some detail: *B. diphtheriæ*, *B. typhosus*, *B. anthracis*, and *Mycob. tuberculosis*. Many of the principles which govern the spreading- and disease-producing properties were there presented, but a general summary in view of the importance, to the human race and to its domesticated animals, of bacteria-induced diseases is necessary.

Koch's Postulates have already been discussed, since they are the basis for the decision whether a disease is of bacterial origin or is due to some other cause. No assumption can be made that an organism is the *cause* of a disease simply because it is present in large numbers in abnormal tissue; the bacillus must induce, by its presence or its products, the pathological condition. A relatively small proportion of the deaths which today occur are the result of the direct attack of bacteria, as can be seen from the following table from the records for 1926 and 1927 of 52 of the large insurance companies of the United States. The deaths probably due to bacteria or viruses are indicated by a star.

The deaths due to bacteria or to some contagious virus amounted in 1926 to 233.8 per 100,000 and in 1927 to 189.9 These figures represent only about 25 per cent of the total number, but it must be recalled that many respiratory diseases are of bacterial origin and that a very large percentage of the deaths from organic disease of the heart were induced in the beginning by bacterial lesions on its valves. Some of the other diseases were probably bacterial. Cancer may yet be shown to be a bacterial stimulus, and the same may be said of meningitis. Taken together, it would be fair to say that probably at least 40 per cent of the deaths recorded by these insurance companies were due, directly or indirectly, to bacteria.

As soon as the fact is established that a disease is induced by an outside organism, at least five facts have to be considered with regard to it: (1) *its portal of entry* into the body; (2) its *spread* through the body; (3) *the type of injury* which it induces, whether local, affecting one organ in the region where it has found lodgment, or general and poisoning the entire body by its secretions; (4) *the means by which its entrance* may be prevented; and (5) *the resistance offered by the body* to invasion by this particular type of parasite.

TABLE SHOWING THE DEATH RATE PER 100,000 PERSONS
(Based on the death records of 52 large life insurance companies)

Causes of death	1926	1927
*Pneumonia	89.9	70.3
*Influenza	25.6	13.6
*Tuberculosis	83.8	77.9
Bright's disease	67.8	63.1
*Diarrhea and enteritis	20.4	15.8
*Measles	6.6	2.3
*Whooping cough	5.9	3.8
Cerebral hemorrhage	56.2	54.3
Organic disease of heart	128.3	126.9
*Scarlet fever	2.0	1.8
*Puerperal state	10.4	10.2
Homicides	6.3	6.2
*Typhoid fever	4.1	4.1
Causes not specified	211.0	206.1
Automobile accidents	16.5	18.1
External causes, not specified	40.8	42.2
Cancer	71.1	72.3
Suicides	12.3	13.4
Meningitis	2.9	3.8
*Diphtheria	5.1	5.9
Respiratory diseases, not specified	11.1	11.4
Total	878.1	823.5

The Portal of Entry.—Each parasitic organism is able to penetrate at some point the barriers which the body has set up in its own defence. The typhoid organism, entering through the mouth, is destroyed or weakened by the passage through the acid juices of the normal stomach, but if it passes this part of the intestinal tract successfully, it may establish itself in the alkaline lower intestine on the Peyer's patches. The diphtheria organism entering through the mouth by the use of some drinking cup, or fork, or food which has been infected, does not pass any further than the throat where it becomes established on the mucous membrane. The anthrax organism must get into the blood stream but entrance can be made at any point. The tuberculosis organism can enter through the intestine, getting into the blood stream and passing to any organ of the body where it may become established, or, it may be inhaled and establish itself in the lungs if they are in an inflamed condition, as may occur in severe colds; or it may grow in some of the glands, bones, or joints. The tetanus bacillus enters the body through some deep wound where it thrives, if the cavity in which it occurs is rendered anærobic by other bacteria which are ærobic and use up the oxygen. The streptococci may enter the body through a break in the skin or they may estab-

lish themselves on some part of the mucous membrane of the throat or the valves of the heart, after entering through the mouth.

The mode of entrance of bacteria through membranes in unknown, but the discovery of stages in the life history of many organisms in which the fragments are ultramicroscopic, or just on the limits of microscopic vision, has led to the suggestion, backed by much experimental work, that the wall of the intestine and other mucous membranes may be passed by such fragments or possibly by the usual rods or spheres.

The Spread through the Body.—Some of the invading organisms never migrate far from the point where they enter, others are carried by the blood stream to all parts of the body and a *general or systemic* instead of a *local* infection results. All parasitic bacteria find some organ or tissue to which they are adapted and on which they grow best; these centers of infection serve as *foci* out of which they find their way to other parts of the body. The typhoid organism, while it is found in the greatest abundance in the Peyer's patches, is spread through out the body by the blood stream and shows in fairly pure condition in the rose spots which appear on the abdomens of typhoid patients. Streptococci may infect the throat or tonsils but be carried by the blood stream to the heart where they may establish themselves and form *lesions* on the valves. The tuberculosis bacterium may spread from one gland to another until a series may become infected. On the other hand, the tetanus bacillus never spreads far from the focus of infection but grows there and produces toxins which are carried by the blood stream to the nerve centers. The diphtheria bacillus is localized in the throat but the toxins there formed poison the cells of the respiratory system and heart.

The Type of Injury Produced.—The injury may be induced by the presence of the organism or its products in the form of toxins or ptomaines. The anthrax bacilli become so numerous in the blood that they stop the blood flow in the finer capillaries of the spleen and liver with the long, delicate threads composed of their cells; they also destroy the blood itself and produce acid in it, probably from the sugar which it contains in small amount. The typhoid organisms do not secrete a definite toxin but apparently induce injury in the body as they are being destroyed and digested by the body cells. In both typhoid and anthrax, the presence of the organisms is indicated by a rise in temperature, known as a "fever." The invasion of the streptococci to form a local sore or a sore throat, results in *inflammation*, a redness and soreness induced partly by the numerous phagocytes which occur at the focus of infection and by the stoppage or slowing of the blood stream. The diphtheria bacilli poison and kill the cells of the throat with which they come in contact, and also induce the exudation of lymph from cells not yet killed. The poison spreads to other cells distant from the throat and does more injury than in the throat itself, as it may cause the weakening and stoppage of

important organs, such as the heart. This general poisoning is also indicated by a fever. The word "fever" is understood to indicate a rise in body temperature apparently induced by increased respiratory activity, a too rapid burning up of the materials in the blood. The tetanus organism, through its toxin, injures the nerve cells but not those of its immediate surroundings. The toxin spreads, after being taken up by the motor-end-nerve apparatus, through the axis-cylinder of the motor nerves to the spinal ganglia on the same side as that on which the injury occurred, passes over to those of the other side of the cord, and finally to the brain. The substance of the brain unites directly with the toxin and from this chemical union arises the definite symptoms of the disease known as "tetanus" or "lockjaw." The blood stream cannot carry the toxin directly to the brain; the passage through the nerve cells is not as rapid as it would be if the blood passed it along almost immediately. In local infections with staphylococci, the injury is entirely localized and the only cells killed are those in or immediately surrounding the lesion.

THE MEANS BY WHICH ENTRANCE AND SPREAD ARE PREVENTED

The body prevents the entrance of bacteria by means of the skin. As already noted, the acid juices of the stomach prevent the passage into the intestine of many of the organisms which are ingested with food. The mucous membranes of the alimentary tract, the respiratory system and the genito-urinary tract offer moisture, proper temperature, and some food to invading organisms and are, as a consequence, some of their principal locations. The proper organism must find its resting place, however, on the proper mucous membrane or nothing will happen, as may be illustrated by the diphtheria organism. If it should be placed in the back of the throat, it might produce the typical symptoms of the disease known as diphtheria, but if swallowed, the alimentary tract would not allow its growth and development of toxin; nor would injury result from its deposition on any part of the genito-urinary mucous membranes. On the other hand, the typhoid organism would not find any food or a location for growth in the back of the throat but must get into the lower intestine in order to make an entrance.

The resistance which the body offers to the invasion and the recovery from the disease are as important as the presence of the organism. Every individual shows more or less resistance to the invasion of the body by foreign organisms and even to the introduction of foreign materials. This is *individual resistance*, but it varies with the condition of the individual as to age, nutrition, fatigue, and sleep. In some diseases, extreme youth is the susceptible period, while in others, extreme old age. If the body is well nourished, is not worked to the point of extreme fatigue, and has a normal amount of sleep, it is very much more likely to be resistant than

if it is fed on poor food, is driven to extreme fatigue, and suffers extremes of heat or cold. Over-feeding might lead to the same result, in an indirect manner, by weakening some of the organs of the body.

In addition to individual resistance, every species of animal has a *varietal resistance* which also varies in many diseases. The monkeys, for example, are very resistant to typhoid-fever organisms, although some of the higher anthropoid apes do contract it, but they are very susceptible to tuberculosis.

In a similar manner, *racial resistance* occurs. The South Sea Islanders and the negroes of the south of this country are both very susceptible to tuberculosis and to genito-urinary diseases, but do not seem to be troubled by typhoid fever. The bacteriologist would explain such resistance by the statement that the body cells of resistant individuals or races are able to destroy invaders when they get into the tissues, or they are able to care for the toxins which they produce, until their body cells have learned the method of destroying them. With pronounced toxin-producing species, the latter is probably correct. The reason for the superiority of one race or individual over another is not clear and science does not offer any visible evidence. The superiority is simply there.

The destruction of the bacterial products and the bacteria themselves has already been discussed in connection with the diphtheria and the anthrax bacilli. The body is exposed at all times to invasion, since pathogenic types must be fairly common in the air, dust, and food. The body is able to care for a limited number by the action of its phagocytes and a real danger occurs only from a *mass attack* at a time when the body resistance is at a low ebb. It is for this reason, for example, that it has been found that about one in six of the married couples where one member of the couple has tuberculosis that the other member has it also, while the usual rate of tubercular infection is only one person in ten. The constant association with the tubercular member of the family at all times, when the body is resistant and when it is susceptible, leads, in the course of time, to infection of the uninfected member. The same danger comes from the use of rooms formerly inhabited by a tubercular patient; the organisms, being very resistant to drying, may remain for months in the dust and, during this time, the succeeding occupant may be at some time in a very susceptible condition.

Susceptibility.—The constant aim of every individual is, therefore, to maintain the body as nearly as possible in a well-nourished condition, to avoid excesses of all sorts, whether overwork, overeating, fatigue, cold, heat, or wet, in order to maintain as nearly an equable and normal a mean as possible. This is especially true where two or more of these weakening influences may appear at the same time, such as fatigue with cold and wet, since such a combination will almost inevitably lead to a cold, if nothing worse.

Recovery and Immunity.—The recovery from an invasion is indicated by the disappearance of the bacteria from the centers where they were producing the disturbance. The bacteria, no longer having the upper hand, are eliminated from the body to a large extent, undoubtedly by the activities of the phagocytes. The body cells have learned the method of disposal of the invaders and if any more appear inside the body, they are promptly disposed of and none of the symptoms of the disease appear. The resistance has been brought to such a point that it is complete and we can say that a person or animal is "immune," a condition which may be maintained for months or years. The immunity which an attack of a disease confers is the most permanent type of immunity, other than a natural immunity.

Vaccination.—In addition to this immunity produced as the result of an attack of the disease, science has been able to develop immunity by a process known as "*vaccination.*" The original vaccination was an attempt to control smallpox and was an intentional infection with the disease, first practiced by the Turks and introduced into England by Lady Mary Montague, the wife of the English ambassador at the Turkish court, who had successfully practiced this transfer of the disease to her own children. The conditions were so favorable for the spread of smallpox in England during the seventeenth and eighteenth centuries that the statement is made that half of the people of the country were pock marked, even some of the kings, such as Charles II, did not escape and 11 of the imperial house of Austria died from it in 50 years. In the Turkish method, the virus from an infected person sick from the disease was carried over to a healthy individual by introducing it into the blood. The disease occurred as in all of its vigor but as the patient was kept warm and comfortable, the serious and often fatal consequences were largely avoided.

Smallpox is one of the infectious diseases produced by an ultramicroscopic organism, usually described as a *living virus.* After the introduction of this virus into the blood, the virus seems to come to the surface of the body in the form of sores or *pustules;* the pustule consisting of a depression in the skin enclosing some pus and covered by a scab. In this pus is contained the active and living virus which may be carried to other individuals.

Edward Jenner (Fig. 174) was an English physician practicing from 1773 at Sudbury in Gloucestershire, a rural community where one of the chief occupations was dairying, the milking being done by young women. Jenner was told, by some of his patients, that the dairymaids who had acquired cowpox through their chapped hands were not subject to a subsequent inoculation with smallpox and, being of a clever and inquiring mind, he investigated the matter. Some physicians who knew of this fact believed that the popular idea was correct while others were

doubtful, but it remained to Jenner to prove it correct. He discovered that the cow was subject to an eruption similar to that found on smallpox patients and that it was possible to communicate the virus from these pustules to human beings in the form of a weakened attack.

The first inoculation of human beings by cowpox took place on May 14, 1796, when a small boy eight years of age was vaccinated with the virus from the hands of a milkmaid (Fig. 174*b*). The experiment was successful; the boy had a case of cowpox and when he was inoculated with smallpox virus on the following first of July, he was found to be immune. Other trials were made by Jenner but it was not until 1798 that he published his results as a memoir. The old method of giving the smallpox virus unweakened by its passage through the cow, was, however, along with the

(*a*) (*b*)

FIG. 174.—*a*. Edward Jenner (1749–1823); English country doctor who discovered that smallpox and cowpox were the same disease and that inoculation with cowpox prevented smallpox; *b*. The first vaccination.

new inoculation, still continued in the smallpox hospitals of England until 1808, when the treatment introduced by Jenner was made the only one. Parliament voted Jenner grants of £10,000 and of £20,000 and he was made Director of the National Vaccine Establishment of England.

The organism in smallpox is weakened by its passage through the cow, in a manner similar to the weakening obtained by Pasteur when he grew the anthrax bacillus for a long time at abnormally high temperatures. In both cases, the organisms producing the disease were inoculated but only after the virulence had been weakened.

The vaccination with the typhoid organisms is of an entirely different type, since the bodies of the dead bacteria are inoculated and absorbed by the patient. The tuberculin treatment of Koch against tuberculosis was an attempt in the same direction but was unsuccessful.

In addition to the use of the organisms, the serum of an immune animal can be used, as is diphtheria antitoxin. The immunity conferred

by such an inoculation is necessarily of brief duration, much more so than if the disease-producing organism itself is inoculated, even in a dead condition. Since the individual upon whom it is conferred has no part in the establishment of such an immunity, it is usually described as *passive immunity* in contrast to active immunity.

Immunity may be summed up as follows:

1. Active immunity:
   - *a.* By an attack of the disease.
   - *b.* By an attack of the disease in a weakened form.
     By passage through another animal.
     By growing at abnormal temperatures.
     By the addition of chemicals.
   - *c.* By the inoculation of the bodies of the bacteria.
2. Passive immunity:
   By the inoculation of the serum from another immune person or animal.

CHAPTER XXXVIII

THE ASSOCIATION OF BACTERIA

Nearly all laboratory experiments in bacteriology (with a few exceptions) have been made with pure cultures. In fact, the pure culture technic is the modern science of bacteriology. A pure culture is inoculated into a special medium, and the growth of the organisms and the products which the organism produces are recorded. The acceleration and, finally, the slowing up of growth in such a culture is the consequence of the accumulation of the excretion products of the cells, since the bacteria cannot remove themselves or the compounds which they form, from the situation where the bacteriologist placed them. The growth of the lactic-acid organism slows when the acidity approaches 1 per cent of lactic acid in the inoculated milk, and in a similar manner varying amounts of acetic acid restrict the vinegar organisms.

As Marshall[4] says:

> A pure culture may tell, if conditions are maintained, of the individual and isolated action of a bacterium, but by no means tell its action when brought into the influential company of another species of bacteria, especially if both are cultivated together in their natural invironment. Analysis and synthesis should go together hand in hand, otherwise our conclusions might be like studying man apart from society in order to obtain his social relations.

In nature, the by-products of one organism are broken up or neutralized by entirely different sets of bacteria, yeasts, or molds. A series of waves, or cycles, of predominant microorganisms follow each other in any food on which they can grow. With the pure culture method such a succession is never possible, and all the results that have been obtained are of rather doubtful value in so far as they are true to natural conditions. The difficulties of the pure culture method are magnified according to the laws of permutations and combinations by the use of two, three, four, or more cultures, although many more than that number must occur in a natural habitat. In fact, the difficulties of making these combinations are so great, and the labor of following them out so vast, that it is doubtful if more than two or three organisms could be studied at the same time.

We know that two bacteria may be *indifferent*, *helpful*, or *antagonistic*, dependent on the use of each other's by-products. We know little about the reactions between cultures growing simultaneously, as relations here are too complicated.

Examples of Various Associations.—The antagonism of organisms can often be observed on plates where a small colony will be held away from a larger colony by its excretion of by-products. The acid-producing types from milk will cut a streak across cultures of one of the dirt organisms from milk, where the two cross each other on an agar plate.

The neutralization of the by-products is one of the methods by which two organisms can be mutually helpful. Many organisms produce acids; many others form ammonium or alkaline compounds. Either excessive acidity or alkalinity is detrimental to growth; for both types a solution more nearly neutral is best. In other cases, the cause is not so simple, but one species may utilize the nitrogenous by-products of the other.

Since milk contains a mixed culture of bacteria, yeasts, and molds, the products obtained are very diverse. Tubes of sterile milk, inoculated simultaneously with two entirely different organisms, *S. lactis* and a peptonizing organism,[4] soured more rapidly than when they received pure cultures of the lactic-acid streptococcus alone. The combined culture $A + B$, as compared to a single-culture inoculation of *S. lactis* alone, gave in one count the proportion of 1,614 to 27, and in another case 1,537 to 271. This helpful action of the peptonizing organism on the growth of the lactic-acid culture may be explained, perhaps, by its formation of weak ammonia compounds and an abundance of readily soluble peptonizing material, the ammonia serving to neutralize the lactic acid and the peptones furnishing a medium rich in nitrogen.

Two intestinal organisms, neither of which alone will ferment lactose with the formation of gas, will, in a mixed culture, develop so much gas that the organism may be taken for *B. coli* and cause confusion in water contamination tests. The combination of organisms destroys the lactose to form acids which are in turn broken down with the formation of gas. Neither organism alone could carry through the series of reactions.[5]

Aerobic and anærobic bacteria often live in cultures where the use of oxygen by the former makes possible the life of the latter. In fact, cultures of anærobic organisms are at times combined with those of ærobes so that the latter may use the last traces of oxygen in the media.

On the other hand, the organism of botulism, an obligate anærobe, may be hindered by the development of acid by bacteria with which it is often associated. The relations here are acidity against an anærobe rather than an ærobe *versus* an anærobe.

The disease-producing organisms are nearly always associated with types which are themselves almost harmless but which may heighten their virulence to such an extent that the results produced by the combination are much more serious than by the pathogenic type alone. Such combinations were found to be especially serious in war-wound infections and have been extensively studied for that reason.

The diphtheria bacillus is nearly always helped, in making serious lesions, by various streptococci, and considerable study has been devoted to such combinations. Other organisms, such as *Staphylococcus aureus*, are more or less antagonistic to *B. diphtheriæ*, overgrowing it in cultures, but are not so successful when inoculated into the throat.

The digestive tract of man and the higher animals is alive with bacteria; it has been compared by Rettger[3] to a true culture tube. The conditions for rapid and continuous growth: temperature and an abundance of food are all here, and every human being excretes daily from 5 to 8 grams of bacteria. A constant association is going on, antagonistic and helpful, among the various species. On account of its importance to health, the intestinal flora has been intensely studied, especially with reference to the effect of the sour milk organism, *Lactobacillus acidophulus*, in controlling the num-

bers and activity of the other groups, especially the putrefactive groups which attack the proteins and form toxic intermediate products.

The Method of Such Investigations.—The usual method seems to be to make simultaneous inoculation of a medium and allow the two organisms to grow side by side. Usually one outgrows the other and becomes the predominant type. Another method that has been used is to grow an organism in a culture medium, such as broth, and then follow this species by a second species in the same medium. This procedure does not allow simultaneous growth but does give some idea of the utilization of the by-products of one bacterium by another.

Many of the early investigations were made by means of colloidan sacs.[1,2] One species was enclosed in a colloidan sac and the other grown in media in a large tube or flask in which the sac was immersed. The by-products of the two organisms osmosed back and forth through the semipermeable membrane of the sac.

The antagonistic influence which the by-products of some of the peptonizing proteolytic bacteria have on disease germs was indicated by the experiments on the water of the Chicago drainage canal where cultures of *B. typhosus* enclosed in such colloidan sacs only lived 3 days in the sewage while in the relatively pure water of Lake Michigan they were alive at least a week.

Such sacs are not ideal for the purpose, and it would seem that other technic might be devised to bring two species into proximity but still hold them distinct, such as cultures separated by very permeable membranes. But, unfortunately, any such mechanical device will not exactly reproduce natural conditions where the bacterial cells of two or more varieties are living and working in close proximity to each other, and the products of one organism are utilized at once by its adjacent neighbor with no accumulation of waste materials to check growth.

Unfortunately, these combinations of two or more cultures of organisms do not seem to have attracted students of the bacteria, although the method would seem to offer one of the most promising fields; it never seems to have appealed to the imagination as do the simpler single-culture studies, but, if there is to be any real explanation of many microbiological processes in nature, some beginnings must be made. In this case, microbiology seems to be waiting for the man with a method.

The literature has been brought together by Frost[1] and more recently, especially on disease organisms, by Holman.[3]

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5. Sears and Putnam, *Jour. Inf. Dis.* **32**: 270, 1923.

CHAPTER XXXIX

THE STRUCTURE OF THE BACTERIAL CELL

The finer structure of the bacterial cell has been the subject of intensive investigation for the last 50 years, but many of the details are as obscure as they were to the students of bacteria in 1876. The anilin stains have been used in staining bacteria since their introduction by Weigert.[63] At the beginning of the seventies histologists were using a few of the dyes, although their technic was confined mostly to hemotoxylin and carmine.[55] The use of the anilin dyes was extended by Koch, Ziehl, Gram, Fränkel, Friedlaender, Ehrlich, and others, so that by 1890, the ordinary technic as practiced in the laboratory today was nearly all worked out. With the advance in the technic of staining bacteria went progress in the histology and cell structure of plants and animals. Many of the facts discovered in staining bacteria were the result of difficulties encountered in staining the tubercle organism and bacterial spores. The small size of bacteria and the still more minute proportions of their organs make them very difficult both to stain and to study under the microscope, after they have been prepared. The older, cruder methods of fixation of the organisms, by the use of heat on the slide or cover glass, have given way to the more modern technic of fixing fluids and anilin dyes, which are best adapted to the various cell organs.

"Dyes are used in bacteriology for four purposes: (1) to make organisms visible, (2) to display their structure, (3) to reveal their chemical nature, and (4) to influence their growth" (Churchman[32]). This chapter has to do with the second purpose: to display the structure of the bacterial cell.

The *cell wall* (with occasionally a *capsule* which seems to be a part of the outer portion of this wall), the *flagella*, the *protoplasm* with its inclusions, which possibly may be divided into the cytoplasm with its inclusions, and *some form of nucleus* constitute the cell organs which have been observed in the bacteria. Not all of these organs occur in the same individual; some have flagella, while on many species they are entirely absent.

THE CELL WALL

The bacteria are enclosed in a cell wall, which may be rigid in form, or may be flexible. The wall is necessarily very thin, since the organisms themselves are usually not much over 1 micron in diameter. In spite of this thinness, Schaudinn[47] separated the cell walls in *Bacillus bütschlii* into two layers (Fig. 175), an *outer* which

at times may become gelatinous, or which may only be the more highly hydrolized zones of an *inner*, which seems to be more in the nature of a cuticle.

The outer layer of many varieties does not stain well in aqueous anilin stains, being apparently too watery to hold the color, and this same layer may pass over in some of the bacteria into a still more watery colloid to form a capsule. Probably many more varieties than are generally supposed, have a capsule under special conditions of growth.[12]

The inner layer, according to Schaudinn, has a mesh-like structure, as seen from above, while, from the side, it appears as lighter and darker areas which alternate. It is not the plasma membrane but lies in intimate contact with the outermost layer of the cytoplasm. The plasma membrane will be discussed in more detail in two other chapters, as it is of much importance in the absorption of germicidal substances which are to penetrate and kill the bacterial cell.

Schaudinn's inner layer may be identical with the layer which retains Gram's stain in Gram-positive bacteria. If this view is correct, it must be very dense, or must form a chemical union with the gentian violet-iodine stain. The identity of Gutstein's "ectoplast" with cell components described by other authors is not clear.[27,28,29]

The possibility exists that Schaudinn did not have a true bacterium but was really working with one of the unknown species of the flagellate protozoa. The true bacteria have a wall so thin that it cannot be differentiated into two layers, in many cases. The distinct, outer, slimy, or gelatinous layer is only marked in capsule- or slime-forming organisms.

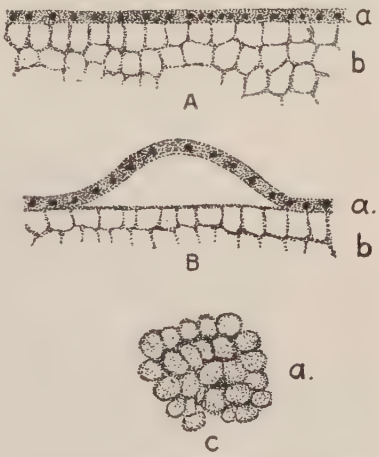

FIG. 175.—Structure of cell membrane of *B. bütschlii;* ectoderm, *a*; endoderm, *b*; in *B*, the outer membrane has been raised up from the vacuolated plasma; *C* is a surface view of the outer membrane, × 5,000. (*After Schaudinn.*)

The same uncertainty exists with regard to all studies on the finer structure of bacteria; in nearly all cases, large and unusual organisms have been selected for study. The limitations of the bacterial group are poorly defined, and some of the organisms which have been investigated as bacteria, may be more nearly related to the protozoa.

The wall is probably not a continuous structure. The flagella protrude through it, since they cannot be regarded as extensions of the cell wall, being contractile and active while the wall is inert and passive. The flagella probably belong to the protoplasm of the organism and are projected through fine pores in the wall, in the same manner that the pseudopodia of some of the protozoans are thrust out through an exterior, hard, siliceous skeleton. Meyer[41] has suggested this arrangement in his diagram of a thick-walled organism equipped with numerous flagella passing to the outside through small pores in the cell wall.

The Demonstration of a Cell Wall.—A distinct cell wall, separable from the protoplast inside it, is somewhat difficult of proof as it is very thin and cannot be differen-

tiated by optical means from the cell contents; it seems to be almost fused to the protoplast and can be separated from it only by the use of very strong plasmolyzing agents, such as the salt solution used successfully by Fischer.[17] Various (coloring) methods to differentiate wall and protoplast have been used, such as that of Grimme[21] who stained *B. tumescens and cohærens*, dried and fixed on cover slips in the usual manner, with a weak aqueous solution of methylene blue or with Gram's stain followed by carbol fuchsin at room temperature. In degenerating cells where the protoplasm was pulled away somewhat from the walls, the cell contents stained dark blue while the cell wall retained the red of the carbol fuchsin. Ellis[15] tried to show the presence of a wall by the use of agents, such as Javelle water, without results, but a 3 per cent salt solution, inducing a strong plasmolysis, followed by a weak staining with carbol fuchsin at room temperature, demonstrated it as separate.

The chemical composition of the cell wall is a matter of dispute and may not be the same in all cases, or, at any rate, the proportion of the various components may vary in different species. Chitin and cellulose are

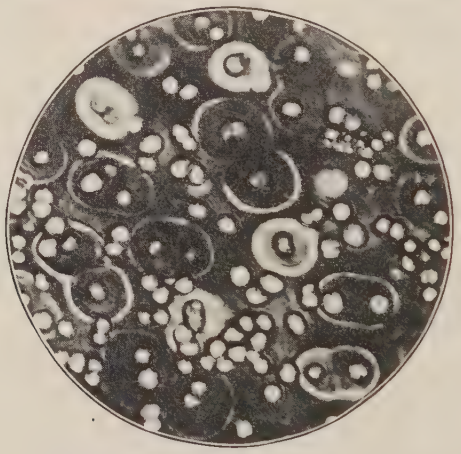

Fig. 176.—Capsules in Azotobacter showing the rings in the swollen outer wall, × 1,000. (*Photograph from Prof. D. H. Jones.*)

probable constituents, although very difficult to identify. From the series of tests made by Viehoever[59] on the cell walls of vegetative cells, sporangia, and spores, chitin seems to be a very probable common constituent. The formula given for chitin is $C_{18}H_{30}N_2O_{12}$, which indicates that it is a nitrogenous compound. The presence of cellulose is less certain, although other carbohydrates, in the outer layers of the cell wall, especially in the capsules are known.

The Outer Layer of Slime; Capsules.—The gelatinous material which forms the outermost layer of many organisms is watery in consistence and difficult to stain, a fairly good coloration being produced (in a solution) in the living cells by methyl violet in 30 per cent alcohol in 2 hours.[41] The usual appearance of this layer of slime in bacterial preparations is that of an unstained zone, or court, around the intensely stained bodies of the bacteria (Fig. 176).

The slime is densest in the proximity of the organisms and gradually becomes more and more hydrolized in each successive layer outward. These layers can be seen

sometimes as zones but the zonation is not as marked in the bacteria as it is in some of the blue-green algæ, such as Gleocapsa (Fig. 177), where the layers are very distinct. The capsule or slime is probably of a carbohydrate nature, although its exact composition is undetermined.

The chemical nature of this membrane was first discussed by deBary: "The cell covering has, in one investigated species, *Sarcina ventriculi*, the same properties as those of the typical plant cell wall in that it is non-elastic, extremely thin, and gives with chlor-zinc-iodide the typical violet color." This slime layer is readily soluble in copper-ammoniac or in very weak hydrochloric acid (5 cubic centimeters 25 per cent HCl in 100 cubic centimeters water) if kept in the latter solution for 8 days at 28°C. (Meyer[41]). These tests indicate its affinity with cellulose. Beijerinck[6] obtained decided tests for cellulose or a cellulose-like compound in this layer on some acetic-acid bacteria: *Bact. xylinus*, and many strains of *Bact. rancens* and *Bact. pasteurianum*. *Streptococcus mesenteriodes*, a marked slime producer, will not show cellulose tests[35] and Buchanan[8] came to the conclusion that the gum which *B. radici-*

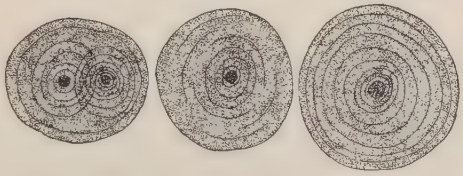

FIG. 177.—*Gleocapsa magna* with zonate gelatinous envelope of the cells, × 250. (*After West.*)

cola forms in such abundance in culture media, especially those which contain sugars such as saccharose, was a dextran. The same author, in reviewing the literature on the subject, points out that dextrans, levulans, and galactans have been detected, and that the substratum upon which the organism is growing apparently determines the slime which is produced. Beijerinck[7] later identified the slime of organisms, such as *B. subtilis*, *B. mesentericus vulgatus*, and *B. megatherium*, when grown in sugar media, as *levulan*—a carbohydrate which they produce from the sugar.

The Flagella of the Bacteria.—The flagella are fine, thread-like appendages which are, in some species, many times the diameter or length of the organism to which they are attached, but which are only 0.05 microns in thickness in the unstained condition. The flagella can be seen with a favorable illumination and a dark field.[40] When stained, the flagellum only forms the core of a mass of material (stain and mordant) deposited around it; its thickness has been estimated by Meyer[41] at 0.09 micron in *B. probatus*.

The flagella seem to be without structure, being simply colloidal, homogenous threads. The active movements which they exhibit are difficult to explain because of this lack of structure; nevertheless, since nothing can be seen inside them, a theory of their movement must be suggested which will cover the observed facts; if they had a firmer central axis with very colloidal flanks, the explanation would be much simplified, but the lack of the slightest internal differentiation makes it difficult. We must assume that the movement is by the contraction and expansion of the sides of the flagellum; a contraction on one side combined with an expansion on the other, bending the flagellum; contraction on both sides shortens it; and expansion on both sides lengthens it. We can only assume, that by a combination of these movements, this

organ can lash the water or serve as a screw propeller to push the bacillus through the liquid, according to the laws of mechanical forces.[45]

Metzner[36] denies that the large flagella in spirillum are homogenous; he sees them as compound structures made up of a number of fibrils or individual flagella which can be separated. The fibrils contract and swing the organ.

Kolkwitz[33] figures the flagella of one of the motile sulphur bacteria, *Chromatium okenii*, as a single whip which may split into its component fibrils (Fig. 178).

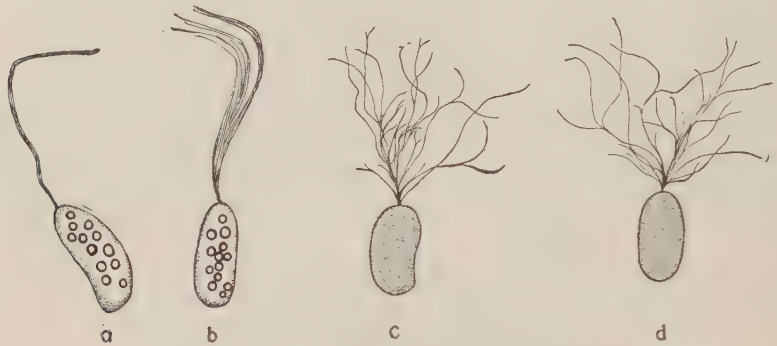

Fig. 178.—The flagella of a sulphur bacterium, *Chromatium Okeni* (*Ehrb.*) Perty. *a* and *b* are cells with droplets of sulphur, flagella undivided or the strands only partly separated; *b* and *c*, are cells with homogeneous contents and the flagella separated into numerous filaments. Loeffler's flagella stain, × 1,000. (*After Kolkwitz.*)

The Attachment of the Flagella.—The flagella, in heat-fixed preparations stained in the usual manner for flagella, seem to spring directly from the cell wall itself, but on theoretical grounds this is impossible unless we assume that some portion of the cell wall is in a very active

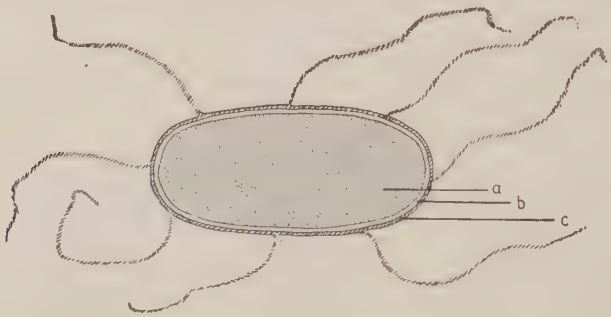

Fig. 179.—Structure of a bacillus (*according to Migula*): *a*, body of the organism; *b*, inner cell wall or endoderm; *c*, outer cell wall or ectoderm from which the flagella arise and through which the materials which enter the cell pass. The flagella are extensions of the ectoderm.

condition. Many early observers unable to see any connection between the flagella and the protoplasm, believed that such a connection was unnecessary; the flagella arose from the outer ectoplasmic layer which normally surrounds bacteria (Fig. 179). Migula says:

The attachment of the flagella to the cell contents or to the denser portion of the cell wall cannot be recognized but the staining of the flagella and of the outer slime layer is exactly the same.

According to this interpretation, therefore, the outer slime layer would be living, nitrogenous in composition, and function as a plasma membrane. Between it and the body of the organism, however, would be interpolated the firmer but non-living cell wall. A similar opinion has been more recently expressed by Zettnow.[64]

The flagella are now believed, by many of the investigators who have studied them, to be attached to the protoplasm of the organism itself, extending through minute pores to the ectoderm and endoderm, as shown diagrammatically by Meyer in Fig. 180.

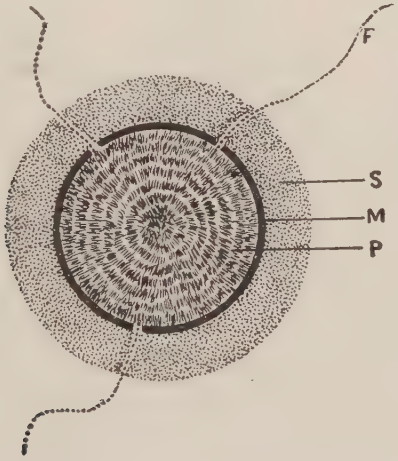

FIG. 180.—Section (diagrammatic) of capsulated bacillus (*according to Meyer*): *P*, protoplasm of cell; *M*, cell wall; *S*, layer of gelatinous material composing capsule; *F*, flagella extending from living protoplasm through the capsule.

Fischer[18] by plasmolyzing the organisms slightly, and then staining them, was able to see a connection between the flagella and the protoplasm of the cell itself. Trenkmann[54] after slightly plasmolyzing *Spirillum undula*, a large organism, states (without figures) that he was then able to demonstrate that the flagella continued as fine threads visible in the area from which the cytoplasm had contracted.

Ellis,[15] by special staining methods which swelled the protoplasm, causing the material to extrude at the ends, demonstrated such pores in some of the organisms which he investigated (Fig. 181). The size of the pores was exaggerated by the methods used, but the connection between flagella and plasma membrane is clear.

The figures of Fuhrmann[19] drawn from plasmolyzed and stained cells of *Spirillum volutans* (in iodine-potassium iodide solution) are still more convincing. The base of the flagellum passes through the cell wall and connects (Fig. 182) either with a granule or with a polar cap of protoplasm. Even after the protoplasm pulls back from the ends of the cell, a small bit of cytoplasm still remains. This polar body is not food material, according to its staining reactions, but seems to be a granule of the

protoplasm itself, imbedded in a still larger mass, which does not stain as densely. The flagella and the nucleus would, according to this view, be in intimate relation through the fine strands of differentiated material which unite internal and external organs. Similar bodies have been demonstrated for the flagellate stages in the algæ by Strasburger and Dangeard. This basal granule, called a "blepharoplast," is believed to function in a manner similar to the middle body of the animal sperm, being associated with the centrosome, an organ very conspicuous in nuclear division, which comes to function later as middle body between the tail of the sperm or spermatozoid and the pointed head.

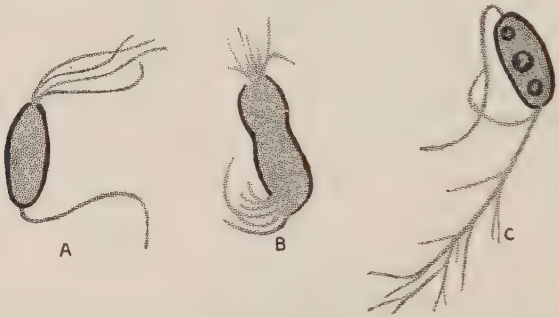

Fig. 181.—Cells of *Sarcina ureæ; A* and *B*, and a diplococcus, *C*, showing connection of flagella with cytoplasm of interior (*according to Ellis*). The cytoplasm has been partially extruded. *A* and *B*, × 2,400; *C* × 4,000.

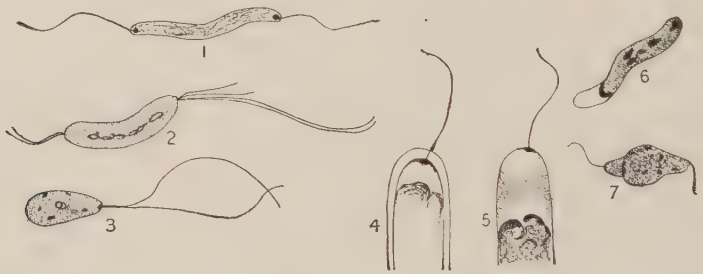

Fig. 182.—Attachment of flagellum to interior of spirillum cell: 1 to 3, organisms treated with various chemicals and stained with iodine in KI solution; 4 and 5, plasmolyzed with salt and stained to show granules inside wall to which the flagella are attached; 6 and 7, old organisms without, or nearly without, flagella. (*After Fuhrmann.*)

The Chemical Nature of the Flagella.—The flagella partake of the same physiological character as the body of the bacteria which bear them and are apparently composed of the same chemical substances. The material in them is in such a very highly dispersed aqueous form, however, that they can be stained only after this degree of dispersion is lowered by coagulation and fixation.[14] The flagella of the typhoid bacillus are so fine that they will pass through the ordinary Berkefeld V filter,[46] after they have been shaken for ¼ hour in order to free them from the cells. The colorless filtrate will agglutinate cultures of the typhoid bacillus and the presence in it of flagella can be demonstrated by staining and

examining under the microscope; the agglutination indicating that they are similar, chemically, to the bacterial bodies.

Cytoplasm and Nucleus in the Bacteria.—Cytoplasm and nucleus in the bacteria must be considered together, as the differentiation of the cell contents into a zone of cytoplasm enclosing a spherical or ovoid nucleus and separated from it by a definite membrane, such as occurs in higher plants, is very doubtful. In preparations of bacteria, made by fixing by heat and staining with an aqueous solution of anilin dyes, the bacteria show no differentiation of any sort, appearing as dense, homogenous bodies. The earlier examinations of bacteria were all made from preparations of this type, so earlier authors insisted that they differed from the usual plant or animal cells by their absence of nuclei, a viewpoint which extended down to about 1888 when various investigators began to find

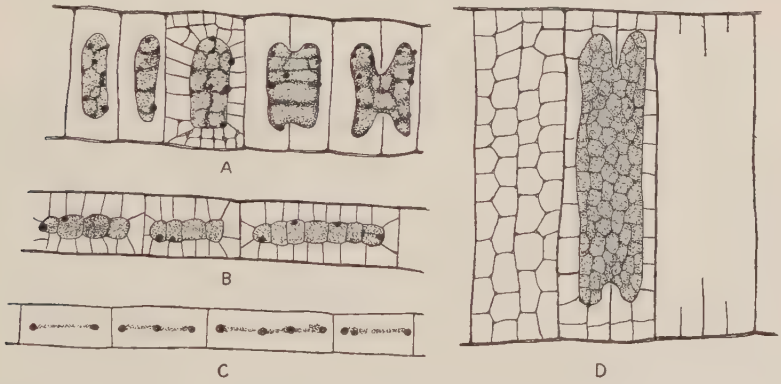

FIG. 183.—Structure of blue-green alga, Oscillatoria according to Bütschli. The central body is shaded. (*After Bütschli.*)

granules and even nuclei in cells after they had been fixed by other methods with special stains. A series of theories have followed this work but the internal differentiation is as much a matter of dispute as it was in 1890.

The difficulties are twofold: the very small size of the bacterial cells, and the lack of any stain for chromatin which will differentiate it from food particles of nitrogenous composition.

The Cell Structure of the Blue-green Algæ.—The nearest relatives of the bacteria are the blue-green algæ, as first pointed out by Cohn in 1872. The cells in these plants are so much larger in size that it may be well to note some of the studies and theories regarding their cell structure before going into detail about those of the bacteria.

Bütschli,[9,10,11] after a careful study of Oscillatoria cells, came to the conclusion that, while they varied greatly as to size and contents, they were all alike in the fact that each had a central body which stained darker than the peripheral portion of the cell, but no definite wall separated the two regions (Fig. 183). Inside this darker

body were other still denser granules and a somewhat reticulated network, the outer portion being vacuolate in accordance with the Bütschli conception of the structure of protoplasm.

Bütschli's work has been confirmed and extended by modern cytological methods, the cells fixed in some of the Flemming mixtures, imbedded in paraffin, sectioned, and stained, by Olive,[42] Gardner,[20] Haupt,[30] and Lee.[34]

According to Olive's interpretation (Fig. 184), the coloring matter is held in the outer layers of the cell immediately under the cell wall. The central portion of the cell is occupied by a denser, but light, staining structure without a definite wall, such as surrounds the nucleus of other plants. Imbedded in this denser portion, which may be regarded as the linin, are a varying number of granules which, staining intensely, are apparently comparable to chromatin. The only structure, therefore,

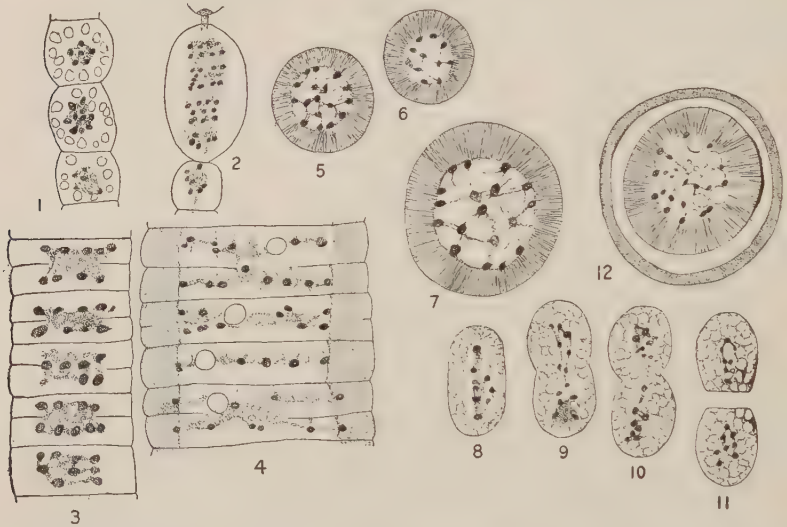

FIG. 184.—Cell and nuclear structure is blue-green algæ: 1, *Nostoc commune;* 2, *Cylindrospermum stagnale;* 3, *Oscillatoria tenuis;* 4, *O. Froehlichia;* 5, 6, *O. princeps* (cross-section); 7, *O. sp.*, cross-section; 8 to 11, *Gleocapsa polydermatica*, dividing; 12, spore of *Cylindrospermum stagnale* dividing, in cross-section. (*After Olive.*)

that is lacking is the nuclear membrane. This membrane may not be an essential structure and may not have developed in these lower forms of life, the diffused type of nucleus being the primitive one. The resemblance of these granules to the chromosomes of higher plants is further emphasized by their behavior during the division of the central body when they line up, apparently divide, and each daughter cell receives approximately half.

Each of the investigators who have studied the blue-green cells has slightly modified Olive's viewpoint: Gardner believes that the blue-green cells have a nucleus but that the divisions are amitotic except in one species, seeming to show a primitive type of true mitosis; Haupt came to the conclusion that the cells of *Anabæna* have no real nuclei but that the darker central body, which occupies the central portion of the cell, functions as a nucleus; and Miss Lee has returned in *Stigonema* to the interpretation that the central body is a nucleus but that it is without a limiting membrane or nucleole and that the divisions are a primitive type of mitosis where no true spindle is formed.

The cell structure of the Cyanophyceæ is of such importance in the evolution of the nucleus as an organ and their relationship to the bacteria is so close that over fifty papers have been written on it. The observations do not vary materially, although the interpretations are not always exactly the same. With the facts about the cytology of the Cyanophyceæ before us, it will be well to return now to the various investigations and the theories which have appeared since 1888 regarding the finer structure of the cytoplasm and nucleus of the bacteria.

NUCLEI AND NUCLEAR MATERIAL IN THE BACTERIA

These theories may be briefly summed up as follows:

1. The bacteria are entirely without nuclei.
2. The bacteria are all, or practically all, nuclei; the cytoplasm is small in amount or entirely lacking.
3. The bacteria have definite nuclei.
4. The bacteria have definite nuclei at least at some stage in their life cycle.
5. The nuclear material of the bacteria is confined to chromatic granules which are scattered throughout the cytoplasm but which function as do chromatic granules in the nuclei of other plants and animals.

1. The Bacteria Have No Nuclei.—This viewpoint, the older and accepted one until 1888, at least, has already been discussed. The methods by which the absence of nuclei had been proved, the fixation of the bacteria in the flame and the use of anilin dyes, were not adapted to nuclear differentiation and make any conclusions based on the older technic very doubtful.

One of the more recent investigators has returned to this view. Alexaieff[1] asserts positively that the bacteria do not have nuclei but that the cells of *B. mitochondrialis* only contain, besides the plasma (cytoplasm), metachromatic granules, mitochondra, and glycogen. The chromatin of other authors is identified as mitochondrial bodies similar to those to be found in many of the cells of plants and animals, but not generally regarded as nuclear structures. These mitochondria are the second elements in the formation of the cells. The plasma is first and primitive; then appear the chondriosomes (or mitochondria of some authors) and, finally, the nucleus. The cell is the unit of structure but it is possible, as in the bacteria, to have cells without nuclei.

2. The Bacterial Cell is Practically All Nucleus.—The theory that bacterial cells are free nuclei with little or no cytoplasm was first suggested apparently by Hueppe,[31] but developed and proof advanced by Bütschli[10,11] in a series of papers. The protoplasm is divided into a vacuolate peripheral layer and a denser inner body which stains more intensely; both regions showing an alveolar structure. Inside the denser body are dark staining small granules. This denser central body with its granules represents the nucleus of the higher forms. The Cyanophyceæ and the bacteria, according to Bütschli, have essentially the same structure. In the larger bacteria (Fig. 185), the central body does not occupy all the cell space, leaving a rather large peripheral zone, but in the very small

bacteria, the outer zone cannot be differentiated, so that the entire bacterial cell is to be regarded as a central body, or nucleus, if it can be called such. This theory has not particularly met with favor, although some other investigators, especially those who have tried to differentiate nuclei in the common forms such as *B. subtilis*, have been forced to somewhat similar conclusions. For example, Wahrlich[62] claimed that bacteria in a young condition are all chromatin with no cytoplasm whatever, simply naked nuclei. In younger cells all material is evenly distributed and homogeneous, but in older ones there comes a differentiation into chromatin and linin threads.

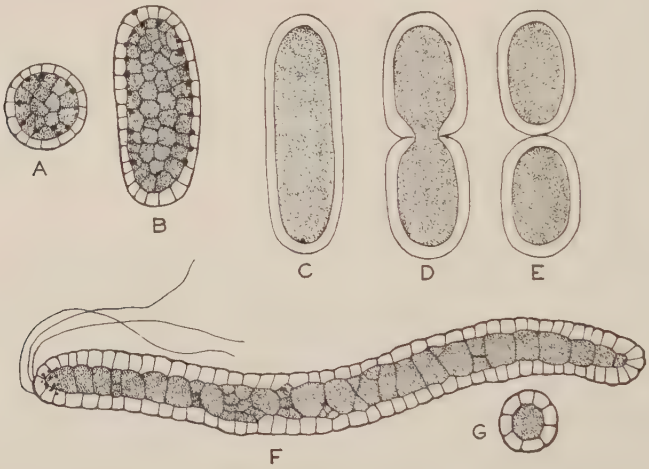

FIG. 185.—Bütschli's conception of structure of bacterial cell. *A* to *E*, *Chromatium okenii* Ehb., large organism of purple-sulphur group, in cross and longitudinal section, and dividing. *F* and *G*, *Ophidimonas jenensis*, member of same group in longitudinal and cross-section. Denser internal body is shaded. (*After Bütschli.*)

3. The Bacteria Have Definite Nuclei.—Schottelius[48] seems to have been the first to claim to have found typical nuclei in the bacteria. By the use of a very aqueous solution of gentian violet for ¼ to ½ minute on cover glass preparations of the anthrax bacillus fixed by heat, he was able to demonstrate a rod-shaped central body; in the coccus forms, this central body was spherical.

Wager,[60,61] without figures, describes a central elongated body which divides before the bacterial cell divides and which he considered to be a true nucleus.

The presence of a real nucleus has been upheld by Arthur Meyer and his students, Vejdovsky[57,58] and Paravicini.[43] Meyer[37] described and figured bodies which he considered were true nuclei in *Bacillus asterosporus*, an organism chosen, as he states, because it was as free as possible from all granules. These bodies corresponding in size to the bacillus could be observed before the spore began to form, either in a living condition by the use of proper illumination or after staining with iodine. After spore formation started in the bacilli, the nuclei were difficult to differentiate.[39] By the use of the formalin-fuchsin method, he[38] confirmed the presence of nuclei in

the cells of *Bacillus tumescens*. His student, Grimme,[21] demonstrated that the bodies observed by Meyer in *B. asterosporus* were not granules of volutin, a reserve food material, but of a different microchemical character. By the use of Flemming's fixing fluid and coloration with iron haematoxylin or Delafield's haematoxylin, Meyer[4]

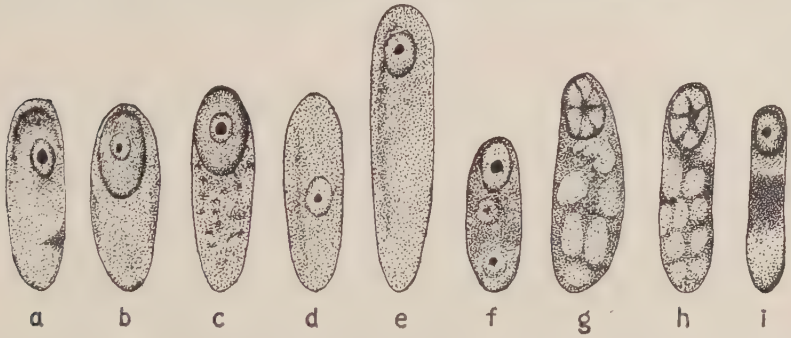

FIG. 186.—Nuclei in the cells of *Bacillus amylobacter* A. M. et Bredeman (*according to Arthur Meyer*): *a* to *c*, fixed in hot water; *d* to *e*, in Flemming's solution; *f* to *i*, young sporangia fixed in Flemming's solution, the nucleus being included in the spore, × 2,500.

demonstrated in the anærobe, *B. amylobacter*, not only a nucleus but also a nucleole and a nuclear reticulum (Fig. 186).

Vejdovsky,[57,58] using a large organism, *Bacterium gammari*, a new species which he discovered in the body cavity and pericardium of a shrimp, Gammarus, demonstrated by the use of haematoxylin and picrocarmine, not only a very definite nucleus

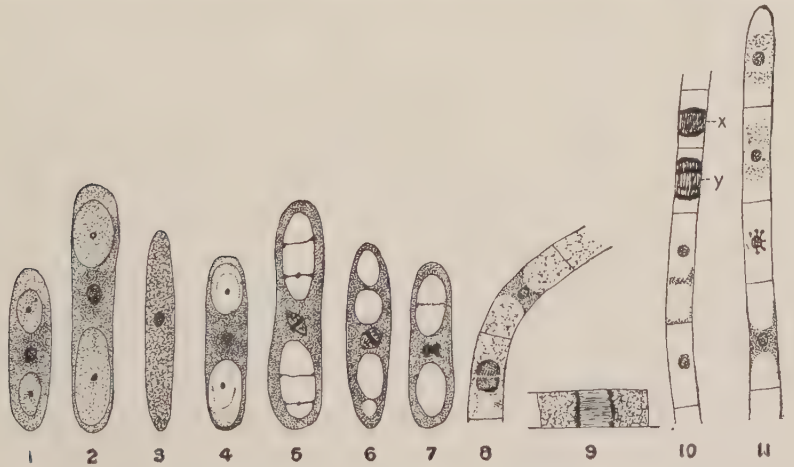

FIG. 187.—Resting nucleus of *Bacillus gammari*, from shrimp: 1 to 4, dividing nucleus; 5 to 7 nucleus at rest and in division of a thread bacterium from the intestine of a worm; 9 and 10, *x* and *y* spindle figures are shown. (*After Vejdovsky.*)

(Fig. 186) but also nuclear divisions with a spindle-shaped figure and a plate of chromosomes. His figures are the most convincing of any of the investigators who claimed to have demonstrated a nucleus.

Prazmowski[44] saw not only a nuclear membrane but also linin and intensely staining masses of chromatin.

4. The Bacteria Have Nuclei at Some Stage of Their Life Cycle.—Amato's work[2] will serve as an introduction to the subject of granules in bacteria and in the blue-green algæ as well. Many students of the cytology of these groups would go with Amato as far as the presence of granules is concerned but deny the presence at any time of a typical nucleus.

Amato is certain that in organisms such as the potato bacteria, *B. vulgatus*, *B. subtilis*, *B. mycoides*, and *Spirillum volutans*, a definite, dark-staining body appears in the *young* cells only (Fig. 188), and then breaks up into fine granules, which dis-

Fig. 188.—The structure of some common bacteria: 1 to 9, *B. vulgatus;* in 1 to 4, the young organism shows a dark central body. This central body diffuses through the cytoplasm and assumes a position near the surface of the cell, 8. In spore formation, these bodies form a cap of denser material which seems later to form the wall of the spores. Similar stages in *B. subtilis*, 10 to 19, and *B. mycoides* in 20 to 28; 27 and 28 show spore formation under abnormally high temperature. (*After Amato.*)

tribute themselves throughout the cytoplasm. The granules may gather (Nos. 5 and 6) in clumps and then migrate to the ends of the bacillus to form densely stained poles as in Nos. 8, 9, 16, 21, 22, 23, and 26 of the same figure. The central portion between these denser ends becomes lighter in color until the spore is completed (Nos. 9, 18, 19, 25, and 26).

Schussnig[43] records similar results on *B. caviæ* in which a differentiation into nucleus and cytoplasm can only be seen just before spore formation.

Amato and Schussnig's results may be questioned with regard to the chemical composition of the bodies observed and considered to be chromatin granules; these bodies may be a food substance which stains like chromatin. Similar granules have been observed by other students of bacteria. Meyer[41] tried to differentiate between

them and the chromatin but chromatin after all is only a special form of stored food and in the bacteria may assume a somewhat different chemical form from that found in the higher plants and animals but still give many of the food reactions.

Awerinzew[4] suggests that the nucleus in the bacteria is not a permanent structure but appears and disappears as do food granules, also enclosed inside them. The nuclei appear as one of the phases in the life history of the organisms but are not found at other times. A somewhat similar view had been held by Vay.[56]

5. The Nuclear Material of the Bacteria is Diffuse, Being Made Up of Small Granules Which Are Scattered throughout the Cells.—Babes[5] found in various bacterial cells, darker stained bodies which he later called "metachromatic granules." Ernst,[16] in the same year, observed densely stained granules, more conspicuous at spore formation when they seemed to gather to form the young spore or to be included in it. Since these granules took part in spore formation, he suggested for them the name "sporogenic granules." All these bodies are now known to the cytologist as "Babes-Ernst granules."

Between the various types of reserve food materials and chromatin, the task of differentiation seems almost hopeless in view of their extremely small size. The cells of the bacteria may contain, according to Arthur Meyer[41] (p. 238), besides fat droplets and particles of *glycogen* and *iogen*, another type of reserve material which he has named *volutin*. Glycogen and iogen (see p. 207) are reserve carbohydrates which are deposited in the vacuoles in the cytoplasm. These compounds are difficult to observe in the ordinary vegetative bacterial cells but are more conspicuous in cells which have gone, or are going, into the spore stage. They may be distinguished by their reactions in iodine: in a concentrated iodine solution, masses of glycogen appear dark reddish brown, while iogen stains blue.

The *volutin* of Arthur Meyer has been studied intensively with regard to its microchemical reactions both by him and by his student, Grimme[21] These bodies, known to Guillermond[22,26] as "metachromatic granules," are of importance as reserve food material. They retain the ordinary stains very readily and Meyer identifies with them the Babes-Ernst granules and probably the blue particles which Löffler and Klebs saw in the diphtheria organisms stained with methylene blue. Other investigators have confused various bodies with volutin, including not only the metachromatic granules but also fat droplets. Volutin occurs in the bacterial cells as small, colorless, amorphous masses imbedded in the cytoplasm. They are so soluble in water that they will soak out of the bodies of dead bacteria into water in 2 to 3 days, or into hot water (80°C.) in 5 minutes. They are insoluble in alcohol, chloroform, and ether but soluble in 5 per cent sulphuric or hydrochloric acids. These bodies stain a dark yellow in iodine solutions, so are not starch-like, but, on the other hand, they are not attacked by trypsin or pepsin, the protein dissolving enzymes. As to their chemical nature, Meyer admits that not much definite can be said but believes that they are combinations of nucleic acid although they are not nucleoproteids.

These bodies are not confined to the bacteria but are to be found in the ascomycetes, yeasts, mushrooms, smuts, oomycetes, and zygomycetes.

With this preliminary discussion of carbohydrate and protein bodies to be found in bacterial protoplasm, it may be well to return to the authors who have found, in the cytoplasm of the bacterial cells, clumps of scattered granules which stained intensely, resembling in their reactions the chromatin of the nuclei of higher plants and animals. A full discussion of all of the investigators who adhere to the diffuse nucleus is impossible but Schaudinn,[47] Swellengreble,[50,53] and Guillermond,[23,24,25] Ambroz,[3]

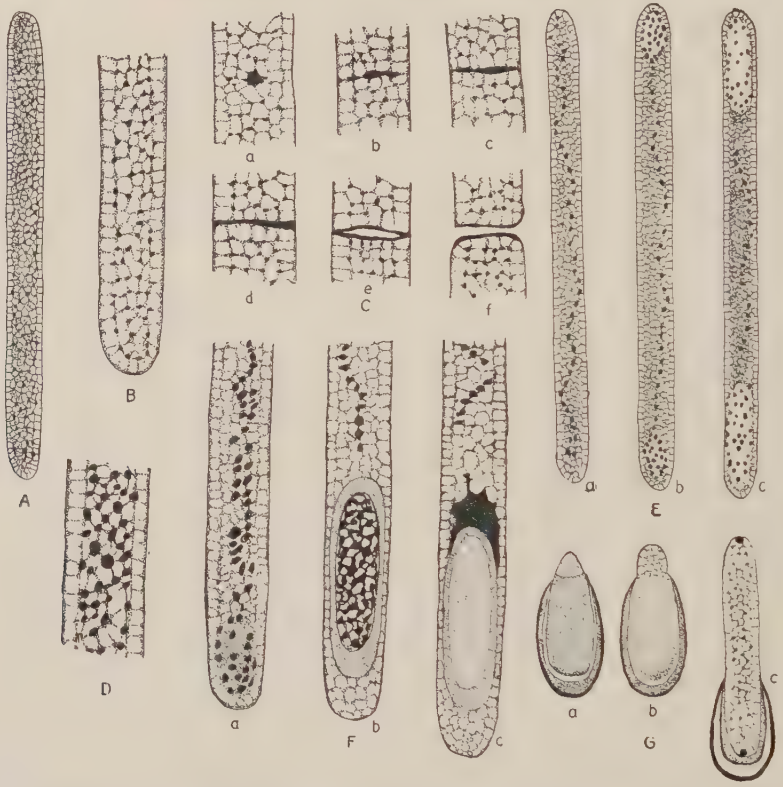

Fig. 189.—Structure, division, and spore formation in *B. bütschlii: A*, normal individual with granules; *B* same, details; *C*, stages in cell division; *E*, stages in spore formation and the accumulation of the granules at the poles; details of spore formation; *G*, germination of the spore. *B*, *C*, *D*, *F*, and *G* × 2,000; *A* and *E*, × 1,000. (*After Schaudinn.*)

and Dobell[13] present typical but slightly different viewpoints of the same general theory.

Schaudinn found a very large bacillus, *B. bütschlii*, in the intestine of the cockroach, very favorable for cytological studies on account of its enormous size (12 × 14 microns). The ordinary vegetative cells have a vacuolate structure, similar to that postulated as a general condition in protoplasm by Bütschli, with granules imbedded in the meshes of the alveoli. At cell division, a wall is put across the elongated cell, dividing it into two portions and roughly segregating into each end approximately a half of these small bodies. No spindle can be observed and no attempt is made to

line them up on an equatorial plate, split them, and give to each daughter cell the half of each granule.

At the time of spore formation, however, the granules form a long irregular thread which at first occupies the middle of the cell and then pinches in two by the movement to the ends of the elongated cell (Fig. 189). The clumping at the two ends of the elongated cell becomes more pronounced by the fusion of these small bodies to form two dark staining masses which later become impervious to stains and highly refractive. Each cell in this organism contains two spores which are released by the breaking down of the sporangial walls. The spores germinate at the ends by the protrusion of a small sack which at first contains no granules; but when they have attained normal length, they resemble the mature organism.

FIG. 190.—Diffuse nucleus of *Bacillus maximus buccalis* (Miller) and the two nuclei of *Bacterium binucleatum:* 1 to 3, resting nuclei with nuclear material arranged in an irregular and often broken spiral; 4 to 6, the irregular spiral and the granules at *x* are splitting in two, lengthwise; 7 to 14, various stages of *Bact. binucleatum;* some show spiral arrangement but the majority have chromatic material arranged as in 8 and 14. The nuclei dividing in 10 at *a*, a spindle seems to be formed at 11*b*. (*After Swellengrebel.*)

Similar results were obtained on a slightly smaller organism, *B. sporonema*, but the axial filament which precedes spore formation in *B. bütschlii* seems to be lacking and the spore is formed by the fusion, after clumping, of diffuse granules. Spore formation by the clumping of granules resembles the older account given by Ernst.

Swellengrebel[50] used *Bacillus maximus buccalis*, a very large rod-shaped organism, 8 to 20 to 27 microns in length and 1.4 to 1.9 microns in breadth, occurring on the outer side of human teeth, as the object for his cytological investigation. The nuclear material in this species is present in resting cells (Fig. 190) as diffused granules, at times arranged in very irregular pattern in finely alveolated protoplasm, or, in other cases, the granules assume a spiral arrangement being connected by darker staining threads, the granules sometimes arranged as a zigzag or spiral. He considers the latter arrangement as typical and all other patterns as degenerate. The nucleus

divides by the spiral splitting lengthwise so that a series of chromatin bodies can be observed arranged in pairs throughout the cell. Such a division of the nuclear material would be more in line with the conceptions of nuclear division, as found in the higher plants, and has met with favor from cytologists, perhaps for that reason.

The following year, Swellengrebel[51] described and figured a somewhat similar appearance in *Bacterium binucleatum*, a smaller organism, 1.2 to 5.1 × 0.6 to 1.2 microns, another mouth organism, although he considered the typical condition here to be not a spiral nucleus but two dark staining bodies. In cell division, each of these

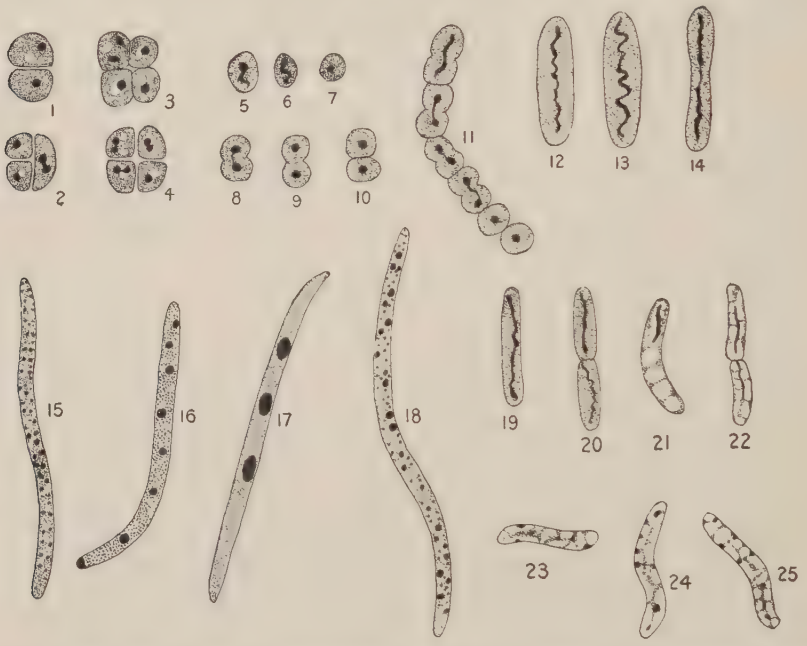

FIG. 191.- The cytology of some bacteria, according to Dobell: 1 to 4, sarcina from toad; 5 to 7 micrococcus from lizard; 8 to 10 similar micrococcus; 11, chain of cocci from same; 12 to 14, bacilli of the spirogyra type where the central body is a spiral strand; 15 and 16, bacilli of flexilis type, 15 with chromodial nucleus and 16 with finely granular cytoplasm and eight large chromodial masses; 17, bacillus of flexilis type from lizard, with chromodial material in three large masses; 18, similar organisms with chromatin in large masses; 19 and 20, bacilli of spirogyra type from lizard; 21 and 22, large spirilla one dividing 23 to 25, similar spirilla with alveolar cytoplasm and chromidal nucleus, × 2,000.

bodies undergoes a division into granules which form 4 nuclei in the dividing cells. Microchemical tests demonstrated to his satisfaction that these bodies were neither fat, volutin, or starch-like in their chemical composition.

Swellengrebel[52,53] answered the objections to his work and maintained his original position.

Guillermond[23,24,25] investigated a number of sporeforming bacteria: *B. megatherium*, *radicosus*, *mycoides*, *asterosporus*, and *alvei* as well as *Spirillum volutans* and is convinced that no nucleus can be differentiated in these organisms, although metachromatic granules are regularly distributed throughout the cytoplasm of ordinary vegetative cells. The cytoplasm is very highly vacuolated, and the granules are held at the vacuolar nodes in the meshes of the cytoplasm. At times these granules collect in the center of the cell to form an irregular mass which may be considered as a nucleus.

especially as it divides with the cell. While Guillermond believes these granules to be chromatin, he offers very little positive evidence other than that they stain well with the ordinary nuclear dyes.

At spore formation, Guillermond's figures are very similar to Arthur Meyer's[41] but the interpretation is very different. The youngest stage of the spore (Fig. 190) is a mass of these granules at one pole of the cell. The granules continue to collect, becoming so dense and large that the ordinary stains will no longer penetrate. Meyer would consider that the nucleus is more readily differentiated just at spore formation than during the period of vegetative growth.

Dobell[13] investigated a series of organisms, obtained from the alimentary tract of various lizards, toads, frogs, and snakes, none of them being grown in pure culture nor their names or characters ascertained. They were fixed and stained by modern methods, however, and the figures which he published are apparently accurate.

In his conclusions as to the presence of a nucleus, he occupies middle ground, for, while he found nuclei in some of the coccus forms (Fig. 191), he is claimed by the advocates of the diffused nucleus, since the bacillus types showed a plate, or a spiral, or zigzag filament, similar to that which Schaudinn had described. In many of them, chromidial granules were distributed throughout the cell or were arranged in a filament, more or less in the form of a spiral (Nos. 12 to 14), his Spirogyra type. At times, too, the chromidial granules appeared at one stage in the life history of the organism but would later clump together (Nos. 15, 16) to form either spherical masses or Spirogyra-shaped nuclei. The spherical nuclei found in the *Coccaceæ* divided amitotically (Nos. 5 to 11) when the cells divided and the long plate of the bacillus types also pinched into two masses, half going into each daughter cell.

His paper was severely criticised by Meyer[41] (pp. 59 to 62) because of the lack of definiteness as to species studied and of microchemical differentiation of the granules, Dobell depending entirely on the staining reaction of these granules as proof that they represented nuclear material.

GENERAL CONCLUSIONS

Is it possible to get any general conclusions from the mass of more or less conflicting evidence regarding the nuclear material and cytoplasm in the bacterial cell? Several difficulties must be pointed out: (1) We are not certain that some of the organisms are true bacteria in the ordinary sense of the word; that is to say, the term "bacteria" may be an all inclusive one embracing not only simpler forms, as *B. subtilis, mycoides*, etc., but very large and complex types, such as the intestinal and mouth organisms. These latter forms may be more nearly related to the protozoa. (2) We are not certain as to the composition of the granules found, as no microchemical test has been discovered that will stain chromatin in such a differential manner that it may be distinguished from all other types of proteins. The volutin granules of Meyer are apparently protein and so very closely related in chemical composition to chromatin that their differentiation is impossible.

The weight of the evidence is that no organized nuclei are present in the majority of the bacteria and that no true mitotic divisions occur. The nucleoproteids are stored in the form of scattered granules in the cytoplasm and are not enclosed in a special nuclear membrane. A clumping and condensation of these granules occurs at spore formation and during

the time that they are enclosed in the spores. In ordinary cell divisions no attempt is made accurately to allot one-half of each granule to each daughter cell. The conditions in the bacteria, are, therefore, essentially the same as found in the *Cyanophyceæ*, although in the latter group the chromatin granules are usually condensed in a more highly specialized body at the center of the cell, while the outer portion of the cell functions as a food forming organ. The central body in the blue-green algæ may undergo a simple form of mitotic division but it is not enclosed in a special membrane shutting it off from the remainder of the cell contents.

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CHAPTER XL

THE THEORY OF DISINFECTANTS, GERMICIDES, AND HEAT COAGULATION

A discussion of disinfectants, and methods for testing them, might lead to the impression that the killing of bacteria by chemicals has all been investigated and settled. The very reverse is true; every theory or method has been questioned by one or more investigators, especially those methods used for testing the relative values of disinfectants (largely in the United States and England) for purposes of standardization for their sale.

The theoretical investigation of the processes of disinfection follows two main lines of thought: (1) *the processes which go on in the individual cell which cause its death*, and (2) *the death rate in a mass of bacteria, known as a bacterial population.* The first process may be compared to the study by a physician of an individual case of fatal sickness: the effect of the drugs are noted and the excretion products determined; while the second would be similar to the studies made by the large life insurance companies on death rates at various ages, occupations, and previous life histories.

THE DEATH OF THE CELL

The death of the bacterial cell may be due to a chemical reaction of the usual type or it may be purely physical in its nature. Theories must be proposed which will explain the union which takes place between the protoplasm of the bacteria which are being killed and the poison which is being combined. Disinfection is a problem in *narcosis* and *poisoning*, both injuring the cell and preventing its normal functioning. Poisoning is generally regarded as an irreversible process, *i.e.* when it has once taken place the protoplasm can never return to the old relations, chemical and physical; narcosis may not be an irreversible process unless carried too far[17].

The Plasma Membrane.—Poisonous substances must first penetrate into the cell if they are to do any marked damage, so the first barrier to cross is the outer layer known as the "plasma membrane." Since the nature of this membrane, as long as it functions, determines the kind of material which enters the cell, any poison must first pass through it before it can act on the protoplasm and nuclear material of the cell itself. Since the plasma membrane is also of so much importance in

cell nutrition, its chemical and physical nature has been the object of much investigation and speculation, and experimental studies have been made on the penetration of substances into the cell through the membrane by dissolving a coloring matter and determining the speed with which it appeared in the various cell contents. If it shows in the interior of the cell, it must have passed through the plasma membrane. Aqueous solutions do not pass into the cell as well as do some fatty, oily substances or the alcohols, although the cell itself is largely aqueous. The study of penetration phenomena has led to the following theories regarding the nature of the plasma membrane:

1. Overton's[29,30] lipoid theory is still much in favor with the students of germicides, although probably not correct (Fig. 192). Since many fat-like substances and the alcohols penetrate the cell so readily, Overton assumed that the membrane itself must be of a fatty (lipoid) nature. It it were entirely of a fatty nature, however, any substance soluble only in water could not pass it, whereas many water-soluble substances do penetrate easily. This led to later modifications to cover the facts.

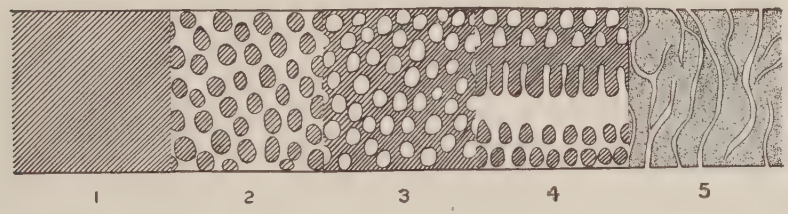


FIG. 192.—The plasma membrane: 1, Overton's lipoid; 2, Czapek's mosaic: 3, Lepeschkin's modified mosaic; 4, Clowe's double mosaic; 5, Ruhland's ultra-filter.

2. Czapek[7,8,9] suggested that the membrane was a mosaic, or an emulsion, composed of an aqueous substance in which appeared lipoid droplets, 2. The cell, coming into contact with fatty material, absorbed it through the lipoids in the emulsion; in contact with aqueous substances, the solvent, being aqueous, unites with it.

3. Lepeschkin[22] reversed the importance of the solvent, making the oil or lipoid substance hold water-soluble material, 3.

4. Clowes[4] suggested that, since lipoid or lipoid-soluble substances seem to be absorbed so readily, the emulsion must be of the type which Lepeschkin suggests, on its *outside* but that on the side next the aqueous protoplasm, the conditions are reversed and the fat is an emulsion in aqueous material, 4.

5. Ruhland[35,36] has suggested that the membrane is a very fine-pored filter through which some substances can pass and others cannot. It not only acts as an *ultrafilter* in a physical sense but selects the material which passes through it, 5.

Ruhland has subjected the lipoid theory to destructive criticism, making it impossible to believe that the plasma membrane is only fat-like. Ruhland's chief objection is that many anilin dyes, such as methyl green, thionin, malachite green, and bismark brown will penetrate into the cells and gradually color their contents, although they are insoluble in lipoids, while victoria blue, night blue, rhodamine *G*, and others, which are practically insoluble in lipoids, behave similarly. These facts must lead to the abandonment of the unmodified lipoid theory in favor of some theory that will explain the entrance of water-soluble substances into the cells.[37,38]

Verkade[46] has attacked the lipoid theory along a still different line. Poisons, such as benzoic, salicylic, and cinnamic acids, are soluble in varying amounts in oils, depending on the composition of the oil itself. Although vegetable oils, such as olive, coconut, and cotton seed, are fairly constant in composition, the solubility of the poisons named seems to vary greatly. The fact that a poison is soluble in olive oil is no indication that it will be soluble in other oils, or that it might pass, through the plasma membrane. The plasma membrane would have to be composed of a lipoid which would unite with *all* oils, and, even then, no assurance can be given that a liquid would pass through it.

It would seem that a useless amount of theorizing has been done about this membrane, only 1/500,000 of a millimeter in thickness according to Loeb[25], but since it occurs in every cell and controls all material which passes in and out, it is of supreme importance. The bacteriologists have taken up to quite an extent the Overton theory and it is true that many of the best disinfectants are soluble only in lipoid-like substances, or if present in an aqueous medium are in the form of an emulsion.

PENETRATION THROUGH THE WALL AND PLASMA MEMBRANE

The fatty impregnation of the wall and body is also important in bacteria, such as the tuberculosis organism, as it excludes all water-soluble disinfectants. On the other hand, phenol, being fat-soluble, is here a valuable germicide. Hailer[17] found that, while the inorganic acids in normal aqueous solutions (such as HCl in a 4 per cent solution) will not kill *B. tuberculosis* in 2 hours, nor a 4 per cent aqueous solution of formaldehyde in 45 minutes, a 1 per cent cresol solution will destroy it in 15 minutes, and a 1½ per cent carbolic-acid solution in 30 minutes. The fatty wall unites with the phenol or cresol and passes it into the cell.

The destruction of the plasma membrane alone would probably not kill the cell, although an alteration in its permeability might, in the course of time, bring about cell death since substances could neither be held in the cell nor prevented from entering. Drastic poisons, such as inorganic acids and their salts, together with some of the organic acids and amido acids, must injure the plasma membrane, but they probably quickly penetrate beyond it, and poison the protoplasm of the cell itself. Yeast cells killed with silver nitrate or mercuric bichloride show the metals in the nuclear material of the cell itself.[40a]

Traube[45] suggests that substances enter into a temporary attachment with the plasma membrane, the nature of this attachment being apparently chemical, and that they are then osmosed into the cell itself. This theory makes the solubility of the disinfectant of importance, for it must pass out of the system in which it is dissolved, such as water, and attach itself to the plasma membrane. The weaker the attachment between the disinfectant and its solvent, the more readily it will leave

that substance and join the plasma membrane. The lipoids of the plasma membrane are especially efficient in removing disinfectants from their solvents, so that the solubility of a disinfectant in a lipoid would be in a way, a measure of its activity; but this is only relatively correct, since we know that soaps and gelatin, which are surface tension depressants, are still not themselves markedly germicidal. It would, however, explain the activity of lipoid-soluble germicides, such as the cresols and phenols.

The surface tension of the outer layer of the bacterial cells is important in growth, morphology, and probably in cell division. A change in surface tension may change the rate and extent of cell division as well as the cell grouping.

Surface tension also may affect the action of a disinfectant. Coating the surface of the bacteria with a soapy substance may partially inhibit the action of a disinfectant, while the reduction of the surface tension may increase the power of the disinfectant. The effect of N/10 HCl may be greatly increased by adding to it a small per cent of alcohol, but not all depressants increase the bactericidal action.[11,12,13,18,15]

The adsorption of disinfectant by the cells of the bacteria is a physical, and not a chemical, phenomenon. Adsorption alone would lead only to the injury of the plasma membrane itself, unless some of the material passed through it and combined with the protoplasm.

THE EFFECT INSIDE THE CELL

The effect of the disinfectant after it gets inside the cell is questionable; it is one of the dark corners of the subject and it is possible that the action is not always the same, being dependent on the disinfectant. The inorganic salts, such as those of mercury, enter into a chemical union with the protoplasm and coagulate it, and Gegenbauer[14] believes that the same thing occurs with formaldehyde, although the chemical union is much slower in the latter case, two days being necessary for its completion. This effect occurs only in weak solutions; with strong ones, the chemical union, although not complete, is sufficient to kill, the speed depending on the rapidity with which it diffuses through the protoplasm.

Some substances are chemically indifferent but may induce a *hydrolysis of the proteins* or a withdrawal of water from them. Among these chemicals are urea, alkaloids, sugars, etc. The withdrawal process results in shrivelling and coagulation, but the reasons for these changes are not clear.

GERMICIDES BREAK INTO IONS, SOME OF WHICH ARE HARMFUL

The basis of much of the older theoretical work on disinfectants was the *ionization hypothesis*, introduced into bacteriology by Paul and

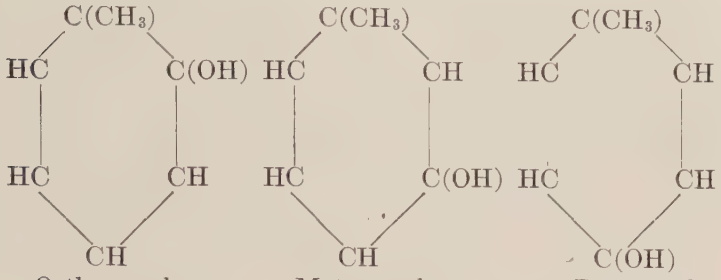
Krönig.[31] One of the ions in a disinfectant is harmful; if the chemical used is an alkali, it is the H- or OH-ions; if of one of the salts of the metals, the destructive ion is that of the metal (or kation), some metals being much more harmful than others.

Paul, Birstein, and Reuss,[32] using a staphylococcus as the test organism, disputed the view that the strength of an acid disinfectant depended upon the number of free H-ions present in the solution. The addition of salt to a weak solution of HCl lowers the number of H-ions in dissociation and if the ionization theory of disinfectants is correct, should produce a corresponding loss in the disinfecting power of the acid solution. As a matter of fact, however, the opposite is really true; since the HCl-NaCl disinfectant solution has many more free Cl-ions, the disinfecting power should be considerably increased instead of lowered. A mercury salt in the presence of NaCl has its disinfecting power much lowered and mercuric nitrate, which disassociates much more than the chloride, does not have nearly the same strength as a disinfectant, although the number of mercury-ions is larger. The explanation offered in the case of the Hg_2Cl_2-NaCl solution is that mercuric chloride forms complex salts, such as $Na.HgCl_4$, which split up into ions in such a form that the mercury is itself really an anion, and does not function as a disinfectant.

Mercuric chloride is one of the most effective of germicides in many cases and its action, as well as that of the other heavy metal salts, has been described as a union of the mercury with the protoplasm causing the coagulation of the latter; in the reaction, part of the mercury is removed from the solution. The process may, however, not be nearly so simple. Gegenbauer[14] claims that the first effect is a hydrolysis of protein due to the acid-ion and that the union of the protein with the salt is only a secondary combination.

Schumacher[40a] has shown that the plasma membrane of yeast cells treated with silver nitrate become semi-permeable, and free phosphoric acid appears in the liquid surrounding the cells; this acid is derived from the nucleic acid of the nucleus, which osmoses out when the plasma membrane becomes functionless. In bacteria[40b] the union of the disinfectant is not with the nucleus, as in the yeast, but with the chromidial granules; the death of the cell occurring when the poison has united chemically with these lipoid-protein bodies. The plasma membrane according to this theory plays only a very secondary rôle; real cell death is the chemical destruction of the nucleus or of the bodies which function as a nucleus.[40]

Effect of Internal Arrangement in Organic Disinfectants.—Phenols, taken weight for weight, are not nearly so bactericidal as cresols. The phenols and cresols, however, also differ among themselves, depending on the arrangement of the groups which compose them. Each of them form ortho-, meta-, and para-compounds, depending on the position of the OH-radical:



Orthocresol Metacresol Paracresol

The three cresols, in the order of their bactericidal efficiency, are meta-, para-, and ortho-, the ortho- being the weakest. The disinfecting power of these chemicals is dependent on a precipitation of the protein colloid; some of the combinations induce this precipitation inside the cells in a weaker dilution than do others, but the cause for this difference, among members of the same composition but different arrangement, is not clear.[33,5,6,41]

Even more striking than the difference between the para-, meta-, and ortho- cresols, and phenols, are those offered by the germicidal activity of isomeric alcohols, phenols, and resorcinols. The higher compounds of these groups are much more effective than the lower ones and the rate of increase in disinfecting efficiency is parallel to that of the molecular weight.

The relative efficiency of such a series of phenols and resorcinols is shown in the following table from Schaffer and Tilley:[39]—

COEFFICIENTS OF THE PARA-PHENOLS AND THE NORMAL RESORCINOLS
TEST ORGANISM, *B. typhosus*

Name	Formula	Phenol coefficient	Molecular coefficient	
			Observed	Calculated
Phenol	C_6H_5OH	1.0		
Cresol	$CH_3 \cdot C_6H_4OH$	2.5	2.9	
Ethyl phenol	$C_2H_5 \cdot C_6H_4OH$	7.4	9.6	9.6
Propyl phenol	$C_3H_7 \cdot C_6H_4OH$	21.6	31.0	31.7
Butyl phenol	$C_4H_9 \cdot C_6H_4OH$	68.0	108.0	105.0
Amyl phenol	$C_5H_{11} \cdot C_6H_4OH$	197.0	344.0	346.0
Hexyl phenol	$C_6H_{13} \cdot C_6H_4OH$	500.0	950.0	1140.0
Resorcinol	$C_6H_4(OH)_2$	0.37		
Orcinol	$CH_3 \cdot C_6H_3(OH)_2$	0.53		
Ethyl resorcinol	$C_2H_5 \cdot C_6H_3(OH)_2$	1.6	2.35	3.32
Propyl resorcinol	$C_3H_7 \cdot C_6H_3(OH)_2$	4.8	7.8	7.9
Butyl resorcinol	$C_4H_9 \cdot C_6H_3(OH)_2$	15.0	26.5	26.6
Amyl resorcinol	$C_5H_{11} \cdot C_6H_3(OH)_2$	47.0	90.0	89.6
Hexyl resorcinol	$C_6H_{13} \cdot C_6H_3(OH)_2$	147.0	303.0	302.0
Heptyl resorcinol	$C_7H_{15} \cdot C_6H_3(OH)_2$	350.0	770.0	1020.0
Octyl resorcinol	$C_8H_{17} \cdot C_6H_3(OH)_2$	400.0	940.0	3400.0

In the preceding table, the molecular weights of phenol and the second disinfectant were multiplied by the phenol coefficient to obtain the "molecular coefficient." The phenol coefficient is expressed in grams; the molecular coefficient on a gram-molecular basis; the latter being, therefore, in chemical terms.

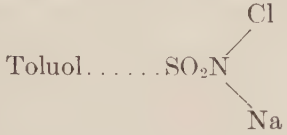
In these series, the molecular coefficient increases a little over threefold between succeeding members, a rate approximately paralleled by the threefold gain in germicidal activity. The possibilities of extremely high bactericidal compounds have not yet been reached in these series, although the phenols, tested reach an efficiency five-hundred times that of ordinary phenol. This high-bactericidal efficiency may not be practical, however, as the cost of production of the high isomers may make their competition with the lower ones impossible.

An adequate explanation for the remarkable germicidal properties of the high-isomeric compounds cannot be offered at the present time. The suggestion[5] that some cresol and phenol compounds coagulate protoplasm in weaker dilutions than do others only pushes back the explanation to the question, What radical in the organic chemical causes the coagulation? The collection of additional data, and the study of the problem of coagulation, may, in course of time, give a basis for a theory which will cover all these facts.

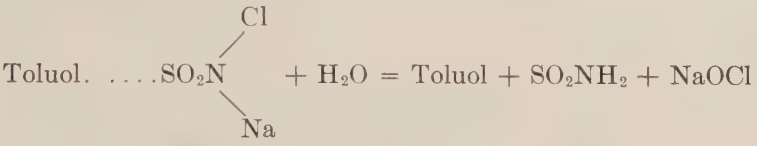
Cell Reactions to Various Disinfectants.—*The complexity of the reactions* is beyond the present skill of the chemist and microchemist to unravel and is probably different with different chemicals. Some disinfectants destroy the cells by combining with the cell acids. Many disinfectants are salts, which are not neutral when in solution. A weak base and a strong acid after combining to form a salt will make a solution which is acid, and, similarly, a strong base and a weak acid will unite to form a basic salt with an alkaline reaction. The dissociation power of the salts of iron and aluminum and of some of the basic colors will serve as good examples. These coloring matters are combinations of strong acids with weak bases which, in solutions, split electrolytically, the free base entering the cell through the lipoid-protein of the plasma membrane, although the salt itself would have been excluded. Once inside the cell, it produces disturbances there, by uniting with some of the acids present.

The presence of the alkali may lower, however, the full effect of a disinfectant, as in the case of alkalized cresols or phenols. The phenols and cresols unite with alkalies to form phenol-and cresol-hydroxides but the germicidal power of the original phenol or cresol is lowered by the combination. Cresol, for example, in a 1 per cent aqueous solution will kill staphylococci in 30 to 40 minutes but the same strength as a hydroxide will require 24 hours. These hydroxides are valuable, however, in solutions where fat is present. The alkali leaves the cresol or phenol combination to unite with the fat, the cresol or phenol is now free to act as a germicide. Such combinations are not only valuable where fat is present in the medium, but also as disinfectants for tuberculosis sputum, as the organism is enclosed in a wall impregnated with a fat-like substance.

One of the substances which came into general use during the war for the disinfection of wounds, Dakin's solution, or chloramine T, depends for its reactions upon a similar combination and electrolytic dissociation. It is an amide of one of the aromatic sulfo-acids where the amido oxide is replaced by chlorine according to the following formula:



which, when dissolved in water gives the following:



The toluol compound is valueless as a disinfectant but the hypochlorite from a 0.1 per cent solution of chloramine T will kill staphylococci in a few minutes (see also Süpfle[44]).[10]

GERMICIDES CLASSED ACCORDING TO THEIR ACTION ON THE CELL

Germicides and antiseptics have already been grouped together according to the strength in which they restrain or kill bacteria (p. 309). Such a table gives no clue as to the action of the various chemicals on the cells themselves, the processes being much more difficult to group, as little is known as to the changes which chemicals produce.

Poisons were classified by Loew[26] according to their method of action, the grouping following, in general, the chemical composition of the disinfectants themselves. This arrangement would mean, therefore, that the effect of the poisons on the protoplasm varies with the different chemical groups.

A. General poisons:

1. *Oxidizing* poisons which destroy the cells by the oxidation of the proteins. Ozone, hydrogen, peroxide, chromic-acid combinations, and chlorine are typical chemicals of this nature.

2. *Catalytic poisons.* These attach themselves to the labile proteins of the protoplasm, not in a true chemical union, but as catalytic agents. Such chemicals as the well-known anæsthetics, ether, chloroform, and chloral, carbon disulphide as well as alcohol, are included.

3. *Salt-forming poisons* include the acids, soluble mineral bases, and salts of the heavy metals. The amino acids of the proteins are able to form salt-like compounds with these metals which may bring about disturbances in the protoplasm leading to death. The chemical combination, which seems to occur, is the replacement of the hydrogen atoms which have been bound up with oxygen or sulphur by a heavy metal.

4. *Substituting poisons* include nitric acid and formaldehyde, also hydroxylamin and phenylhydrazin. The first two of these chemicals attack the amino groups while

the last two attach themselves to the aldehydes. They are poisonous in even very small quantities.

B. Special poisons:

1. *Poisons only which attack certain proteins,* since they are not able to attach themselves to proteins in general, are themselves proteins and include the bactericidal substances secreted by the cells together with the poisons, ricin, abrin, etc.

2. *Poisoning by organic bases,* such as strychnine, quinine, solanine, cocaine, morphine, atropine, and neurine. This is one of the darkest regions of toxicology; some act on some organisms and not on others, so that we can draw no general conclusions.

3. *The indirect poisons* include carbon dioxide, the sulphites and thiosulphites, neutral iodide, salts of nitric acid, and nitrogen combinations. Some of these work by affecting the respiration; some by breaking up potassium iodide and depositing iodine in the organism.

This classification, although made before the modern studies on toxins and poisons, is very suggestive.

THE DEATH RATE IN A BACTERIAL POPULATION FOLLOWING THE USE OF A GERMICIDE

The other phase of germicidal study which has received attention in recent years is *the rate at which bacteria die* when placed in a germicidal solution.

The two methods which are in use for testing disinfectants are: (1) the suspension of the organism in liquid, and (2) the drying of the organisms on bits of thread or cloth before applying the disinfectant. The suspension method is essential to any study of the rate of death at succeeding intervals of time. A little of the suspension is removed at each interval, 1 minute, 5 minutes, 10 minutes, 15 minutes, etc., and plated in agar to determine how many are living at the time. A little of the disinfectant is carried over in this process, but this is made as small as possible so that it will not affect the growth on the agar. The drying of organisms on threads or bits of cloth is likely to weaken or kill a number of the vegetative forms, but this procedure is of value in testing of the much more resistant spores.

The cultures which are made by transfers from a disinfectant at the various intervals of time must be made on the best possible medium, since the organisms are often injured by the disinfectant sufficiently to prevent their growth on a less favorable substratum. This influence of the medium upon which they are grown after treatment was first discussed by Gruber[16] and in recent years has been investigated by Süpfle and his assistants[43] and Lange.[20] The addition of dextrose to the bouillon into which they are transferred seems to aid many of them to recover from the effects of the disinfectant, while they die in ordinary media without sugar.

The weakening effect of the use of a disinfectant such as copper, sulphur dioxide, sulphurous acid, sulphites, and chlorine has been noted particularly by the water bacteriologists (Sturgis[42]) from counts made on water after it had been treated with these chemicals. The phenomenon is known as "delayed development of colonies," since the colonies will not appear in the usual 2 or 3 days at 20°C., a 7-day count being necessary in order to get the real number. The probable cause of this slow development is that the vitality of those bacteria which survive is so weakened by the action of the chemical used; furthermore, traces of the chemical agents remaining in the bodies of these microorganisms weaken them, so that they develop very slowly when placed on nutrient media. This suggestion, if correct, would mean, further, that any pathogenic organisms which slipped through the disinfection process might be so weakened that it is very doubtful if they could ever cause disease.

The Curve of the Death Rate is That of a Monomolecular Reaction.—The first studies which were made on the death rate after application of a disinfectant were by Paul and Krönig.[31] The method used was to add as nearly a definite number of bacteria as possible to a culture medium and then apply one strength of disinfectant.

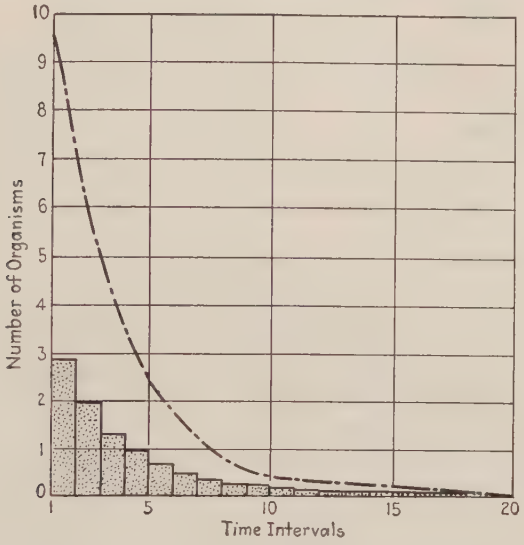


FIG. 193.—Curve of death of organisms in germicida solution. The curve represents number of survivors; shaded blocks, number killed; the two are reciprocal; the curve is that of a monomolecular reaction. (*After Reichenbach.*)

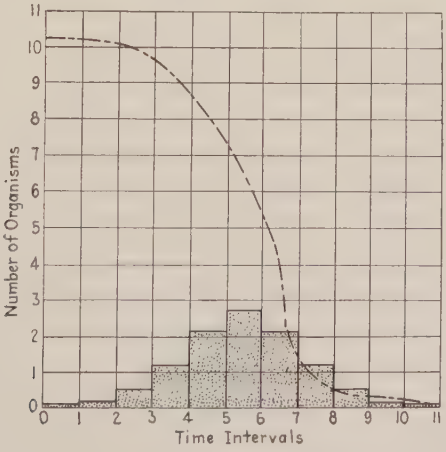


FIG. 194.—The hypothetical curve if the organisms were average resistant and mostly killed in the middle period, shaded areas representing deaths.

At the end of a definite time, the number of survivors was determined by plating out a little of the liquid. This operation was repeated until it was found that all of the organisms were dead. These authors obtained many data but drew no general conclusions from them. Madsen and Nyman[27] from their own data and from those already

furnished by Paul and Krönig showed that the reaction took place in such a way as to follow the curve of a monomolecular reaction. If the number of organisms in the beginning is a and the number of unkilled cells at any following time is x then in the very short time dt, the uninjured number dx, will be proportional to the value $a - x$ according to the formula:

$$dx = (a - x)dtK \text{ or } \frac{dx}{dt} = K(a - x)$$

in which K would be a constant dependent on the temperature, disinfectant, organism, etc. The differential quotient $\frac{dx}{dt}$ one can designate as the *reaction speed* and K as the constant of the *speed of* the *reaction*. The number of organisms still living is always proportional, at any time, to the total number in the medium at the beginning, as shown in the curve in Figs. 193 and 194; the larger the numbers at the beginning, the larger the number of survivors at any interval.

The curve of the survivors falls away sharply at first and then flattens out until all of the cells are killed. The dead at any interval are represented by shaded blocks in the corner, greatest at first, and become numerically smaller and smaller as time passes, until the last most resistant survivor succumbs.

Three Factors in the Death Rate.—Three factors have been found to be involved in the destruction of bacteria by germicides: *the constant of rapidity, the exponent of concentration, and the temperature coefficient.* The constant of rapidity varies with each disinfectant used, and must be determined by experimentation; the exponent of concentration should be predicted after a little preliminary experimentation; while the temperature coefficient should follow the coefficient of a chemical reaction. These factors are so much influenced by the nature and kind of the bacterium and by the medium in which the reaction occurs that its effect also must be determined.

Miss Chick[1,2] added many details of interest to the general laws discovered by Madsen and Nyman. The reaction seems to take place, according to her theory, exactly in the same manner as a chemical reaction in a test tube, one reagent being the disinfectant, the other the protoplasm of the bacterium. As with most chemical reactions, heat increases the speed with which it is carried on, so that heated disinfectants act much more quickly than cold ones. A temperature coefficient can be established, as well as a concentration of disinfectant coefficient, as seen in Fig. 195, which shows the effect of temperature on the time required to kill *B. paratyphosus* in two strengths of silver nitrates. Mercuric chloride seems to unite temporarily with the protoplasm of the organisms and if some substance is added (such as a soluble sulphide) before the action has gone too far, the vitality of the unkilled organisms can be restored. Apparently the toxic substance formed is of such a nature that its poisonous effect is destroyed by the sulphide. This toxic substance will first prevent growth, and then kill, the stoppage of growth being a much easier reaction to induce than the death of the organism.

The earlier view of Miss Chick that the death of the bacteria in a germicide follows a simple curve, resultant from the action of the poison on the bacterial cells, has been somewhat modified by some of the recent workers (Winslow and Falk, 1920). The shape of the curve is dependent on *two* sets of factors and not on *one* alone: (1) the velocity with which the physical chemical changes go on in the cells themselves, and (2) the variations in the resistance of the individual cells to the toxic substance present.

The latter factor is a variable one. Falk and Winslow,[11] using *B. coli* in various solutions of NaCl and $CaCl_2$ (both weak disinfectants), consider that, while there are some deviations in the curve which they drew from that of a monomolecular reaction, in general they are very similar (Fig. 196). In the part of the curve where the solu-

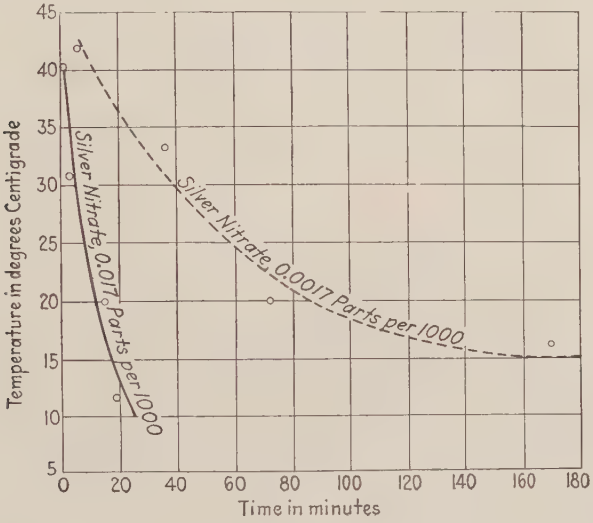


FIG. 195.—Effect of temperature on death rate in disinfection. (*After Chick.*)

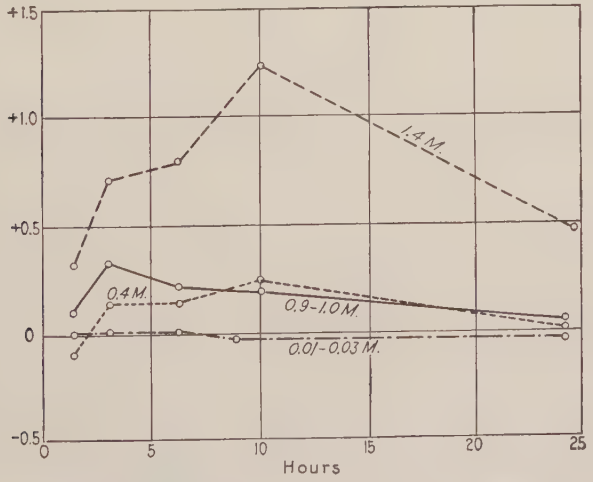


FIG. 196.—Variations in the value of the constant K, with *B. coli* in NaCl solutions of various molarities. (*After Falk and Winslow.*)

tions used were very weak, they showed a stimulative effect and the indifferent and poisonous effects appeared only with higher concentrations. The salts, being very dilute, were allowed to remain in contact for some hours with the organisms, so that the reactions could be more easily studied. The K_1 value obtained for the various

hours with salt was as follows: 1 hour, 0.37; 3 hours, 0.57; 6 hours, 0.88; 9 hours, 1.22; 24 hours, 0.48.

Varietal Resistance of Bacteria.—If the curves obtained either by Madsen and Nyman or Miss Chick were entirely correct, the possibility of determining the value of a disinfectant would be greatly simplified since time, temperature, and concentration could be controlled and, after the constant for any germicide had been determined, the values of any particular dilution could be, theoretically, plotted as a curve. Unfortunately, the *varietal resistance of the organism in the culture varies* too widely and no two organisms react in the same manner to a poison (Reddish[19]).

Deaths are Most Numerous at First.—As was already indicated, the majority of the bacteria are quickly killed in a germicide, the resistant numbers being relatively small (Madsen and Nyman;[27] Miss Chick[1] and Reichenbach). The impression seems to be common that the majority of the organisms have an *average* resistance and die after the culture has been exposed some time. If this were true, the bulk of the deaths and the sharp decline would appear at its middle (Fig. 194). But all the facts which have been collected indicate that *the bulk of the deaths occur very soon after the organisms are placed in the disinfectant,* as the curve in Fig. 193 indicates.

The disinfection time is also dependent on the *number of organisms* present in the culture at the beginning of the experiment, a fact expressed numerically as follows: if the numbers in the beginning are changed *geometrically*, the time in which they are destroyed is changed *arithmetically*. The curve in Fig. 193 shows that if the numbers originally present, about 9,500, were reduced to half, the whole curve would be moved down and the point where the last organisms are killed, instead of being at 20, might now be 17 or 18.

Density of Numbers of Organisms Affects Death Rate.—Another fact about disinfection, developed by Lange,[21] is that *the density of the suspension* of the bacteria will influence the time of death for the entire mass, the organisms apparently exerting a protective influence on each other, when they are present in great numbers in the liquid in which they are being destroyed. Similar results can be obtained in killing the organisms by heat; the bacilli in 3 drops of a culture of *B. coli* added to 1 cubic centimeter of water and then to 5 cubic centimeters of water are killed in 30 minutes at 60°C.; but 1 cubic centimeter of the same culture, added to 5 cubic centimeters of water, required 60 minutes at the same temperature to complete their destruction.

The curious fact is also brought out that the addition of dead organisms will have almost the same effect in weakening the disinfectant or lengthening the disinfecting time as will living organisms.

This fact is shown in the following table:

TABLE SHOWING THE EFFECT OF ADDING LARGE AND SMALL AMOUNTS OF BACTERIA (STAPHYLOCOCCI), EITHER DEAD OR ALIVE, TO SOLUTIONS, OF PHENOL, ALONG WITH THE USUAL RATHER SMALL INOCULATIONS OF LIVING ORGANISMS
+ INDICATES GROWTH

Transferred to agar slants after	3 minutes	5 minutes	10 minutes	20 minutes	30 minutes	45 minutes	60 minutes	2 hours	3 hours	4 hours	24 hours
A. 3 drops of the living culture in 6 cubic centimeters disinfectant.											
Phenol, 0.5 per cent	+	0+	0	0	0	0	0	0	0	0	0
Phenol, 0.35 per cent	+	++	++	++	++	++	++	1(+)	0	0	0
B. 1 drop of the living culture in 6 cubic centimeters disinfectant.											
Phenol, 0.5 per cent	++	++	+	30	0	0	0	0	0	0	0
Phenol, 0.35 per cent	++	++	++	++	++	++	++	++	++	++	0
C. 3 drops of living culture in 1 cubic centimeter of the dead culture added to 6 cubic centimeters disinfectant.											
Phenol, 0.5 per cent	++	++	+	18	0	0	0	0	0	0	0
Phenol, 0.35 per cent	++	++	++	++	++	++	++	++	++	+	0

The explanation offered by Lange is that either the bacteria themselves change the disinfectant chemically so that its action is weakened or that it may be partially adsorbed by the increased surface offered by the addition of so many organisms. This explanation, however, does not suffice for the effect which the addition of masses of organisms has on their survival in heated water, as similar effects occur in the death rate from heat.

The Most Resistant Individuals Are Left to the Very Last in Every Disinfectant Trial.—Can this quality of resistance be transmitted to their descendents so that the form of the curve would be altered at the resistant end? Experimental work shows that, although the progeny of a bacterial race may be very resistant, the later descendents lose their resistance and have exactly the same degree of it as ordinary cultures with a curve of the form indicated in Fig. 193. This fact suggests that while resistant bacteria are exactly the same as the others they are in a different stage of development, and that this phase of growth is a resistant one. This resistance, which appears in culture, being *developmental and not inheritable* is not the same type of resistance which different strains of the same organism would show in their reaction to di infectants or to heat.

The Curve of the Death Rate and the Phenol Coefficient.—The phenol coefficient which has been used extensively in England and the United States for the practical testing of commercial disinfectants is not a true constant but varies (1) according to the variety of bacteria used, some organisms being very susceptible to phenol while others are not, and (2) according to the concentrations of the phenol and of the unknown disinfectant compared. The curves for the various concentrations of phenol

are not parallel but change their form so that they are not, in themselves, a constant. Phenol coefficients determined for organisms suspended (1) in distilled water, or (2) in distilled water with protein dissolved in it (as in broth) give two coefficients which are not usually the same.

Lockemann has suggested dividing disinfectants into eight groups based on chemical resemblances, one representative of each group to be used as the basis for comparison with the others of this group, but not with other groups. The comparisons must be made on a series of trial organisms with various substrata and with various concentrations of the solutions. The point in this suggestion is that it is impossible to compare two disinfectants as unlike as corrosive sublimate and phenol; it is like comparing an axe and a razor; both will cut, but the operations are very different.

Reichel[34] has proposed a curve for each disinfectant, this curve expressing the relation between the concentration and the time required for the death of the organism. The curve would be an expression of the equation

$$T \times \text{Conc.}\, n = K$$

The time of application multiplied by the concentration gives a constant. If the concentration is 1, the curve would then be a simple hyperbola; if it is greater than 1, the time for the death of the organisms increases more than the concentration, while if it is less than 1, the time for killing them remains behind the concentration. The curve which he recommends for use is that for formaldehyde, a fairly simple one. It must be noted, however, that any system involving the construction of such curves would be only another expression of the phenol coefficient, and just as dependent on the bacteria used, the temperature of the reaction, and media employed. It is very doubtful whether it would be any marked advance.

It may be stated that, while the phenol coefficient is probably incorrect in most cases, it gives a very fair estimate of disinfecting value. It is useless to put too much confidence in it with reference to special organisms, as, for example, the tubercle bacillus, but for ordinary bacteria it is reasonably accurate; enough so, that a statement of coefficient value should carry some significance, although, theoretically, very far from the entire truth.

THE HEAT COAGULATION OF PROTEINS

The coagulation of protoplasm is an indication of the death of the individual cell and its organs. As seen under the microscope, it is accompanied by the formation of granules. Life and growth are only possible in a cell during the time that the materials which compose it are in a colloidal phase, due perhaps to the fact that particles in such a condition present an extraordinarily large surface for adsorption of materials. If, however, some chemicals, such as alcohol or acids are applied, the coagulation granules which appear cannot do the work that the same material carried on when in a dispersed colloidal phase. If coagulation and granule formation have not gone too far, the granules, however, may be dissolved by the cell enzymes. Lepeschkin[24] would make this property the indication of cell death; as long as the cell can dissolve these granules, it is alive; but when the enzymes, capable of gelatinizing them

are destroyed, the cell is really dead, *i.e.* death is really a destruction of these essential enzymes.

Lepeschkin[23] divides the process of heat coagulation into two phases: (1) a reaction between the hot water and the protein; (2) agglutination, a process which is dependent on the relation of an electrolyte to temperature.

Water is necessary for the reaction, and experimental work has shown that bacteria die much more rapidly in a liquid than in a solid medium and that vegetative cells are much more readily destroyed than spores.

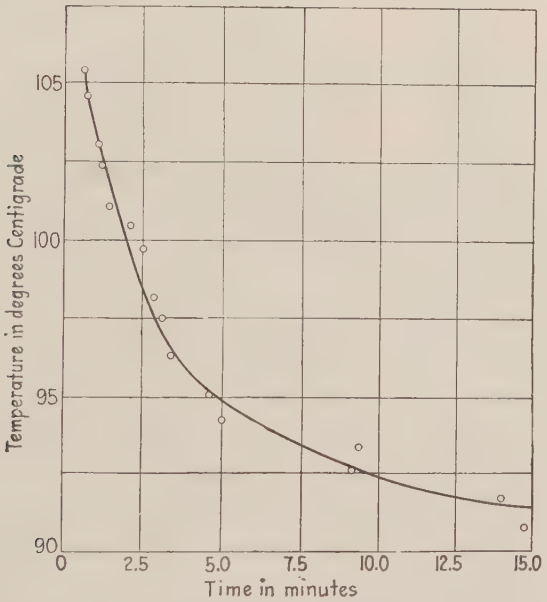


FIG. 197.—Sterilization of anthrax spores with saturated steam at different temperatures. (*From Ballner's results.*)

These facts have led to the general acceptance of the Lewith (p. 177) theory that the resistance of spores is due to their low water content, as compared to the vegetative bacteria (Fig. 197).

Percentage of Water Present Affects Coagulation.—Since coagulation is a precipitation reaction in which the water plays an important role, combining with protoplasm to produce an insoluble compound, the less water present, the less danger of coagulation. Michel and Wichman have made the observation that dry albumen crystals can be heated to 150°C. without change. In very dilute solutions of egg albumen, the process is a very rapid one at first (Chick and Martin[3]) but falls away in the same form of curve as that assumed by a monomolecular reaction, as the following table shows:

Coagulation of 1 Per Cent Solution of Crystalline Egg Albumen

Temperature of bath, degrees Centigrade	Time, minutes	Amount filtrate analyzed, cubic centimeters	Weight coagulum, grams	Residual albumen, milligrams per cubic centimeter
71.1	11	13.5	0.1326	9.823
	37	17.2	0.1162	6.756
	62	11.7	0.0607	5.187
	90	18	0.0850	4.722
	146	19.5	0.0634	3.251
	280	23	0.0487	2.117

The acidifying of a solution of protein quantitatively hastens the reaction of coagulation. On the other hand, the addition of a small quantity of alkali reduces the coagulation rate. The first accretions of a dilute acid do not have much effect, but, as more and more acid is added, the coagulation rate is speeded up, so that the average rate seems to be proportional to the H-ion concentration of the solution. The general conclusion (Chick and Martin[3]) is that heat coagulation is a reaction between water and protein in which temperature accelerates the process, an acceleration which can be further hastened by the addition of acids, although the acids themselves do not seem to take a part in the reaction. The addition of alkali to a solution in which bacteria are being heated seems to lower the time required, especially on some organisms such as *B. coli* (Myers[28]). *B. coli* being naturally adapted to an acid environment, heat and alkalinity work together to weaken and destroy it.

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CHAPTER XLI

MODERN ATTACKS ON THE CONSTANCY OF SPECIES

INTRODUCTION

Bacteriology, between the years 1895 and 1916, had settled down to the solution of minor problems involving the methods of technic and observation which had been established between 1875 and 1890. If anything morphologically at variance with these methods was discovered, it was considered to be simply an abnormal development of bacteria and was dismissed as such. The rigid conception of the fixity of species, as formulated by Cohn and Koch, was in the ascendant and any attempt to question it was ridiculed. While many new facts were discovered during this period, no attempt was made to question the basic principle that a bacterial species was just as permanent as that of any animal or plant, and admitted only a narrow variation at any stage in its development; a rod-shaped form could never transmute into a coccus, nor a coccus grow into a rod.

Fig. 198.—Carl Wilhelm Nägeli (1817–1891). Noted in microbiology as the opponent of the theory of the unity of bacterial species.

Between 1875 and 1880, two schools of bacteriology were battling for supremacy in Germany; one of these was headed by Nägeli (Fig.198), professor of Botany at Munich, included the Buchners, Billroth, and others, and had its expression in the textbook on bacteria which Nägeli[26] issued; the other school was headed by Cohn, professor of botany at Breslau, who was ably assisted by the rising young Koch. It will be noted that, at this time, bacteriology was still in the hands of the botanists. As the importance of the disease-producing types became more and more emphasized, the specificity of one disease caused by one organism made a belief in the constancy of the species a necessity and it became a fundamental dogma of the Cohn-Koch system as they laid down its principles. The disproving of some of the flagrant errors of authors

such as Billroth, cast doubt on the work of the whole Nägeli school and Koch and Cohn won a decisive victory.

The cardinal principal of the Cohn-Koch theories was the permanence and unity of species. Slight changes might be induced by variations in the culture media, or by the temperature at which the cultures were incubated, but such fluctuations would disappear if the normal conditions were restored. On culture media, one species of organism had an approximately definite size and form.

In 1916 and 1917, two revolutionary papers appeared and started a long series of investigations. These two papers were on life cycles in the bacteria by Löhnis and Smith[22] and on invisible organisms antagonistic to the bacteria by d'Herelle.[19,20] Scattering observations had been made along both these lines by investigators during the years when bacteriology was dormant but here an open and careful attack was made against the older tenets of monomorphic bacteriology by experienced laboratory workers.

The Five Main Lines of Attack on Species Constancy.—Since these dates, current bacteriological literature has contained an increasing number of pages on (1) breaking up of organisms known to originate from a single organism (*dissociation*); (2) sudden appearance in old cultures of new strains (*mutations*) of organisms with entirely different properties; (3) dissolution (*lysis*) of organisms in cultures induced by some unknown factor; (4) variation in size in artificial cultures; (5) variation in size and structure at various stages in an artificial culture to form a recurring cycle.

The observations which have been made along these five lines are so diverse that the various threads are somewhat difficult to follow, as they involve not only cultural and microscopic work but, also, filterability, with physiological and serological reactions. Any understanding of modern bacteriology, however, must be incomplete if it does not include these topics since *they are fundamental, both theoretically and practically.*

MICROBIC DISSOCIATION

(The sudden appearance of new stains as mutations)

Dissociations, Variations, Saltations, and Mutations.—Old and apparently stable cultures of some of the most carefully studied species of bacteria suddenly give rise to new strains of very different physiological, morphological, and serological properties. These appearances have already been described (p. 173) in *B. subtilis* which may split into three types and in *B. coli* (p. 200) which throws off a lactose fermenting type. Such phenonomena are known to some investigators as "*dissociations*," to others as "*inconstants*," "*variations*," "*saltations*," or "*mutations*."

According to the interesting theory of deVries, new species of plants and animals arise by sudden and pronounced transformations of pre-existing species. Such variants, since they arise at a single jump without all of the intermediate gradations between the old and new, as postulated by Darwin, were named, by deVries, "*mutants*." The mutation theory seems to have especially appealed to microbiologists; the intermediate gradations are so small in these organisms that all the emphasis has been laid on the more noticeable changes.

Variation inside the Species.—In addition to these recognizable different groups ("elementary species"), every species of plant and animal varies within itself both *morphologically* as to shape, size of parts, arrangement and number of organs, and *physiologically* as to enzyme secretion (determining the type of food available), chemical composition, and excretion products. The problem of variation inside the species, and the limitations of such variations without a new species arising, is a very complex one in the higher plants and animals, but even more difficult in organisms microscopically small and without many of the distinguishing characters which we associate with the more complicated multicellular plants and animals.

Variation in the bacteria is very easy to induce, as was early shown by the work of Buchner,[9] on the hay bacillus, or by a number of investigators on color changes in organisms, such as *B. prodigiosus*, by the use of heat or chemicals. Such induced variations, however, are not permanent, as the organisms revert to their usual form or color if returned to the former environment. In fact, permanent variations in bacteria or any of the lower colorless plants are very difficult to induce, and the records of such inductions are not plentiful.

Variation and the Gene Theory.—Such mutants in nucleated plants and animals, in order to be permanent, must be different in their internal hereditary material (chromatin) from the species from which they arose. Some new units (*genes*) must have arisen, some old ones have been lost, or new combinations must have occurred. If such transformations did not occur in the hereditary substance, the variations were transitory, being simply *modifications* which would disappear when the old conditions returned. Other new species arise by the crossing of preexisting species to form a hybrid which is like neither parent, being an intermediate form with some of the characters of each of its progenitors.

Variation and Chromatin in the Bacteria.—The bacteria seem to be without true nuclei or true divisions of the chromatin. The question naturally arises, therefore, can there be true mutants in such an aberrant group?[21] According to the particulate theory of heredity, certain characters associated with small concrete bodies, known as "genes," are built up in a very definite pattern into chromosomes. The bacterial cell with its lack of definite or ordered chromatin structure, as far as it is visible, would seem to offer an impossible condition to the appearance of real mutants and the elaborate schemes, worked but by the modern school of heredity, do not seem to apply here, since there is no physical basis for them. Moreover, if modifications should appear in the chromidial masses inside the cell (genotypic changes), no new combinations could come about from sexual crosses, since sexual stages are denied in the bacteria

by most authors, although affirmed (as is a definite nuclear apparatus) by Enderlein,[15] Mellon,[24,25] and Almquist.[2,3]

Modifications and Mutations.—Some authors class these variations in the bacteria not as special inheritable properties but as developmental and physiological phenomena involving only the cytoplasm; others[21] refuse the use of the term "mutation," to such variations and simply call them "permanent modifications." For permanent modifications, the gene (and its attendant character) need not be invoked since they apply to the cytoplasmic changes and not to the chromidial substance. Such a permanent modification might be heritable and be continued into the offspring, or, it may be lost, if the conditions unfavorable to its development should arise. Jollos' "permanent modification" seems not to have been accepted by other authors who pay no attention to the difficulties involved in the gene hypothesis and still use the word "mutant."

Dobell,[13] after a very complete review of variations in the lower organisms, limits the terms "modifications" and "mutants" as follows: "Let us suppose that a given bacillus is colored red under normal conditions. By growing it and its offspring upon a new medium they become—let us suppose—colorless. If the organism and its descendants, when transplanted again into the original medium, are again found to be red, then the change (loss of color) is a *modification;* if, on the other hand, they are found to be permanently colorless, then the change is a *mutation.* By far the greater number of variations described in bacteria are of the former type." Beijerinck[6] also adheres to the use of the word "mutant" for bacterial changes which are permanent, although he at one time had suggested the word "variant."

Winslow[31] divided variations into (1) fluctuations, (2) mutations, and (3) impressed variations but Adami (1918) believes that they are all of the same order.

The word "mutations" seems to express the same idea in the lower forms as it does in the higher plants and animals, if the gene difficulty is disregarded, so it will be used in Dobell's sense, or with the same meaning as Jollos', "permanent modifications."

Mutants may arise either:

1. By a change in the media of physical environment.
2. On the same media or in the same environment.

1. *Mutation by a Change in the Medium*

B. coli and *B. typhosus* exhibit much variation in their fermentation of the carbohydrates, but *B. coli* will ferment lactose with the formation of lactic acid while *B. typhosus* will not. This reaction gives a rather certain means of differentiating between two otherwise very similar species and has been extensively used in connection with various indicators in the media for the detection of the acid formed by the much more numerous *B. coli.*

Massini[23] discovered, on plates made from cases of enteritis, not only typical *B. typhosus* colonies but some abnormal ones as well, the latter being especially noticeable, since the cultures were made on Endo's medium, an agar containing lactose, fuchsin, and sodium sulfite. Sodium sulfite renders the medium colorless, but if a lactose fermenting organism, such as *B. coli,* grows on it, its acid converts the colorless fuchsin into the typical bright red dye. *B. coli* colonies are, therefore, colored red; *B. typhosus* colonies, colorless. The organisms plated out by Massini showed only colorless colonies, but, after some days of incubation, small red nodules began to appear, until some colonies contained inside them as many as 200. Since these nodules, on plating, gave cultures which would ferment lactose, when grown on Endo's medium they were always red (from the lactose fermentation); a character

which held permanent. The colorless colonies of the original culture had given rise to a red colonied variety, or in other words, *B. typhosus* had changed into *B. coli*, so far as fermentation of lactose was concerned. The white colonies, if diluted and plated out, would continue to undergo the same transformation—white colonies which, after a time, developed red colonies inside them. Since mutants continued to form in the cultures, Massini called his organism *Bacillus coli mutabile* but regarded it as a *typhosus* species with the ability to change into a *coli* species.

This epoch-making work was followed by many other investigations on the sugar transformation possibilities of the *B. coli-typhosus* group. Cultures of organisms similar to those of Massini have yielded at least four different races with permanent characters. Burri[10] isolated a race which he called *B. imperfectum*, which was unable to ferment either sucrose or lactose. Among the *imperfectum* individuals, however, there appeared, at times, other individuals which were able to split sucrose. This strain was regarded as a mutant of the *B. imperfectum*. The character of sucrose fermentation, if once acquired, might be considered to be a permanent character, and the species in which it had appeared was named *B. perfectum*. The fermentation of a sugar is the result of the secretion of an enzyme; *imperfectum* species do not secrete this enzyme; *perfectum* species do, but all of the former have the *potentiality* of such a secretion and, although it may be latent, the proper stimulus will gradually call it from a latent into an active condition.

This variant, like nearly all of them,[1] is the result of a new environment. The descendents of an organism are not all alike; some vary in one direction and some in another. The individuals, whose variations are favored by the new environment, multiply; those variants that are harmed are retarded in their growth, so that, in a short time, individuals favored by the environment overgrow the others, and a culture with new properties is evolved. The potentiality of variation must be in the cells themselves, the environment only calls out certain phases by favoring them.

Physiological variations induced by changes in the substratum are usually climinated, if the cultures are again returned to the old conditions, as illustrated by Bergstrand's experiments.[7] From *B. coli*, grown on Endo's medium, two distinct strains could be isolated. These strains, so different in cultural characters, agglutination tests, and motility that they seem to be entirely separate and distinct, revert back to the original strain if allowed to rest.

Not many records of permanent physiological variations are tc be found; organisms seem to be so labile that they can meet almost any environmental condition, and use almost any carbohydrate as a source of food, although some time may be necessary for the transformation. The composition of the cells is not always the same, both organic and inorganic elements which compose them may vary within rather wide limits.

By variations in the composition of the medium, Dawson[12] grew *B. coli* with sulphur varying between 0.0 to 0.14 per cent; phosphorus (P_2O_5), 0.92 to 4.24 per cent; calcium (CaO), 0.05 to 2.66 per cent; nitrogen, 2.11 to 6.22 per cent; coagulable protein, 0.97 to 5.544 per cent; carbohydrates, 1.00 to 4.00 per cent; and cellulose-like sub-

stance, 1.00 to 2.42 per cent. But in spite of these differences in composition which involved also an alternation of the biological and physiological characters, such as agglutination and the sugar splitting enzymes, no observable morphological mutants could be detected and the new properties were readily lost.

B. prodigiosus has been the subject of considerable experimentation to modify its red color production. Growth of this organisms in the 37°C. incubator will prevent the development of color, and this loss of color is permanent, unless the cultures are returned for some time to very low temperatures. Wolf[33] found, however, that by growing the organism for some time on media containing small quantities of mercuric chloride, intensely red colonies and also pure white ones appeared. Other salts, such as potassium bichromate, copper acetate, cadmium nitrate, and nickel nitrate would also induce white races, but in all cases they revert when returned to the ordinary media. Some salts, such as cadmium nitrate, intensified the color reactions so that extremely red colonies and pure white colonies would appear on the plates, but the modifications were not permanent.

Pathogenicity is Not a Permanent Character.—Koch was the originator and defender of the fixity of bacterial species, especially of the pathogenic types. In fact, the acknowledgement that harmless species could adapt themselves to a parasitic mode of life would have been fatal to his germ theory of disease, and he was able to disprove and discredit Buchner's claims (p. 260) that the hay bacillus could be converted into the pathogenic, *B. anthracis.*

Loss of pathogenicity is quite a common feature of laboratory cultures, but acquiring this property is more difficult. Adami[1] quotes, with approval, the transformation of *B. mycoides*, a harmless soil inhabitant, into a pathogenic organism which resembled *B. anthracis*, by Thiele and Embleton.[30]

The principle upon which they depended was the phenomenon of *anaphylaxis.* After a culture of an organism, even a harmless one, has been inoculated into an animal, the body is susceptible to successive introductions of the same organism.

Thiele and Edwards, after cautiously accustoming cultures of *B. mycoides* to body temperatures, raising the incubation heat a degree at a time, inoculated some of the dead bacteria into animals. This inoculation was absorbed, the animals were sensitized by the absorption of the proteins, so that they could not destroy more of them at once. A living culture, accustomed to 37°C., was now inoculated which lived in the animal tissues and could be recovered from them. They had become so virulent that they would kill a guinea pig in 12 hours and had taken on many of the morphological characters of the anthrax bacillus.

Loss of pathogenicity may be due, at times, to the overgrowth of pathogenic strains by non-pathogenic ones. The pathogenic diphtheria organism is often mixed in the throat with non-virulent (diphtheroid) types from which it is difficult to distinguish.

Crowell,[11] from a culture of a virulent diphtheria organism, isolated by the Barber pipette method, a single individual. Cultures made from this individual developed strains that were pathogenic; other that were not. Pathogenicity is not a permanent character, since an individual might give rise to descendents that showed its pathogenicity while others did not.

The impressing of non-pathogenic characters on *B. anthracis* by Pasteur is one of the classic examples of the inheritance of acquired changes. Not only did these

strains, raised at a high temperature, lose their pathogenicity but they also lost the ability to form spores.

Spore production, or lack of production, has been extensively studied for variations. The normal race, according to Eisenberg,[14] is the sporogenic one, the asporogenic race is a mutant from it, produced by growing the cultures with frequent transfers. He claims that, if 5 to 20 transfers be made at short intervals on glycerine agar, the spore habit will be lost entirely and never regained. Pure sporogenic races may be isolated by heating cultures until all, except the spores, are left, when these spores will give rise to a spore-bearing race with no non-spore-bearing mutants appearing.

Morphological mutations or dissociations occur at times, although, in general, form characters seem even more fixed and constant than physiological ones. The work of Barber[5] on *B. coli* has already been discussed as well as that on *B. subtilis* by Soule[29] and further examples may be found cited by the latter author and by Hadley.[17,18]

While *B. coli* is a very variable organism and easily modified, investigators have found difficulty in impressing permanently morphological variations induced by physical or chemical changes in the environment. Scales[28] produced unusual forms by growing it on media with a high osmotic pressure (6 per cent salt in the ordinary nutrient agar), or, induced coccus-like varieties on high nitrogenous media (nutrient agar with egg added). Any of these morphological changes would disappear, however, as soon as it was transfered to ordinary laboratory media. This experience is a common one, not only with *B. coli* but with almost all bacteria. The influencing of form and size from *without* seems to be a slow process; the changes which arise that are permanent originate largely within the organisms themselves and have their expression in their variation.

2. *Variations and Mutations not Due to Change of Environment*

Not all mutants are due to a change of environment, although the majority of those observed are of this type. Sectors of a bacterial colony may be markedly different in appearance from the other portions of the same colony (Fig. 199). Transparency and surface elevation of such a sector may vary from the original, but the organisms composing it remain constant with different morphological, cultural, and physiological characters.

Such colonies would be at once under suspicion of being mixed, but, as cultures made from a single organism, isolated and examined before transfer, produce them,[27] such growths must be considered true mutants (Fig. 199).

The relation of such mutants to the degenerate lytic spots in colonies of *B. pyocaneus*, or the d'Herelle phenomenon on intestinal organisms, is not clear. In the latter instance, however, the bacterial cells disintegrate entirely and do not reproduce after they have become affected. The possibility may be suggested that a variant is the result of lytic action which is incomplete, never destroying or injuring the cells, simply inducing the changes in them.

The relation is also not clear between these mutants and the Löhnis-Smith series, which appear in old cultures, and of which any phase may be fixed and continued as a pure culture.

The fact also must be pointed out that the cultural difficulties in making the plates have led some investigators[8] to doubt much of the mutation work.

Mutation in Other Bacteria.—The problem of mutation in bacteria is too complicated to discuss in all details for each species but the series of papers by Eisenberg[14] can be studied by those interested, as this investigator has taken up the mutation problem on a series of common and typical bacteria including *B. anthracis*, cholera vibrio, *B. prodigiosus*, *B. violaceus*, *B. fluorescens*, *B. pneumoniæ*, *B. typhosus* and *B. coli*. The great variability in colony formation and cultural characters, as shown by 14 common species, have been analyzed by Baerthlein[4] and the impossibility of making a description which will fit all the variants is made so evident that every student of microbiology should understand that the older dogma of fixation of species' characteristics has many exceptions and limitations.

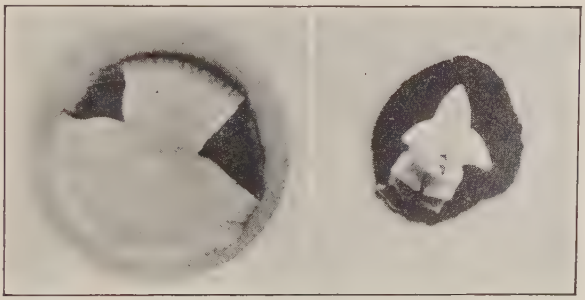

FIG. 199.—Mutation in a bacterium, a saprophyte accompanying the soft-rot organism in turnips. In 1, the typical colony shows two variant sectors (shaded); in 2, the variant has surrounded the parent organism which only appears at the middle (lighter color). (*From Nirula.*)

THE ORIGIN OF NEW SPECIES IN NATURE

The difficulty, by any of the ordinary cultural means, of establishing in a short time, in the laboratory, new and stable variants, is clear from the work which has been cited. The strains of an organism which come up in pure cultures isolated from natural surroundings, are manifold. These variations may indicate that long growth under natural conditions fixes certain characters in a manner that we cannot hope to rival by a few weeks' or months' growth in the laboratory, or (and this seems much more reasonable from laboratory experiments) some factors, unknown to us, cause organisms in their natural habitat to mutate. Beneficial mutations help organisms possessing them to survive, and a strain with these properties becomes the dominant one. In a pure culture, the descendants of this organism will apparently have a well-marked set of characters, but if an isolation is made from another source, the characters may be very different. As Winslow[31,32] emphasized, no organism should be described from a single isolation; the more strains available for comparison, the more nearly correct the description will be.

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CHAPTER XLII

MODERN ATTACKS ON THE CONSTANCY OF SPECIES

(*Continued*)

THE TWORT-D'HERELLE PHENOMENA

(Bacterial autolysis)

One of the most interesting observations of recent years, one of both practical and theoretical importance, is that of d'Herelle[10] that bacteria may be "attacked" by still smaller bodies and the cells "dissolved" (*lysis*). This observation, if correct, implies that beyond the range of the microscope there exist invisible bodies capable of dissolving the visible bacteria.

The entire subject of lytic action is a field of investigation and controversy with much disagreement as to the origin and nature of these bodies and the action on the bacteria. Any statements made must be regarded, at the present time, as only tentative, as new experiments may render them incorrect, or at least only partially correct.

The size of these ultramicroscopic bodies has been estimated to range between that of a protein molecule and of the smallest of the bacteria, composed of 10,000 to 20,000 molecules. Their discoverer[11] (pp. 274 to 275) estimated them to be of about the same size as a molecule of serum globulin, which has been calculated to be between 20 and 30μ in diameter, so minute that they would pass any ordinary bacterial filters, and even fine gelatin and colloidin membranes. Later estimates[8] indicate even a smaller diameter, possibly only 5μ.[3]

Earlier Observations on the Dissolution (Lysis) of Bacteria.—Hankin[9] had investigated the waters of the Jumna and Ganges rivers in India long before the work of d'Herelle. These rivers are frequented by pilgrims who bathe in the waters but do not contract the intestinal diseases which are often to be found in some parts of the Far East. The water of these rivers has been reputed to have bactericidal properties, and the experiments show that bacteria are very readily destroyed by their action,the cholera vibrio being particularly susceptible. The Jumna river water, just below the city of Agra, contains 100,000 bacteria per cubic centimeter, but 3 miles further down the numbers have been reduced to 90 or 100. The cholera vibrios are destroyed by a stay of 3 to 4 hours in the water. No case of cholera has ever been traced to the use of this water. After the d'Herelle discovery, this instance has been fre-

quently cited as an example of the bacteriophagal action of the natural waters of a river.

Twort's Earlier Observations.—Several years before d'Herelle announced his discovery, Twort[15] described a virus which appeared on agar colonies, and which indicated strongly a similar action. From a glycerinated-vaccine virus plated out on agar, he obtained colonies, some of which appeared white and opaque and others transparent. Under the microscope, the transparent colonies, when stained, appeared to be composed of fragments of bacteria. But if one of the white, opaque colonies were inoculated with a little of the material from one of the transparent colonies, the opaque colony appeared to dissolve at the point of inoculation, and the transparent area to spread throughout it, except for certain small islands which remain unaffected. The liquid obtained by passing some of the transparent colonies through a filter had a similar effect, dissolving the denser colonies, even after dilutions of millions of times.

d'Herelle does not believe, however, that the transparent colonies are related to his bacteriophage, as they behave somewhat differently. In Twort's experiments, the small islands of unaffected bacteria grew back over the bare places where the culture had been dissolved by the destructive principle. With the d'Herelle bacteriophage, a subsequent growth was reported never to appear.

The dissolution of the bacteria (lysis) occurring in both cases however, they have been grouped together in the present chapter, as the observed differences may not be essential. d'Herelle has investigated his phenomenon so much more completely, however, that most of the observations given here are derived from his publications, especially his book.[10]

THE ISOLATION OF THE BACTERIOPHAGE

These ultramicroscopic corpuscles, named by their finder *Protobios bacteriophagus* (*Bacteriophagum intestinale*) were obtained by the following technic:

The dejecta from a patient affected by diarrhea was mixed with water to make a very liquid culture which was then filtered through a bacterial filter to remove all the organisms. The dejecta contained both the bacteriophage and the bacteria; the filtrate was free from bacteria. If some of the filtrate were added to a culture of the diarrhea organisms, they disappeared. The bacteriophage was sufficiently abundant in this filtrate that a little of it added to a broth culture of the dysentery bacillus would clear it up in 12 to 18 hours at 22°C. followed by 2 to 3 hours at 37°C., while similar tubes to which it had not been added would remain cloudy from the bacteria. If the bacteriophage were not quite so strong or numerous, as might occur after a very high dilution, the tube of broth might still remain partly cloudy, indicating that some of the bacteria had escaped. The broth must be of neutral, or slighty alkaline, reaction

(pH 7.8) in order to obtain the best results. The effect of the bacteriophage on cultures on solid media is especially striking (Fig. 200), since the growth which has been affected by the bacteriophage is all "dis-

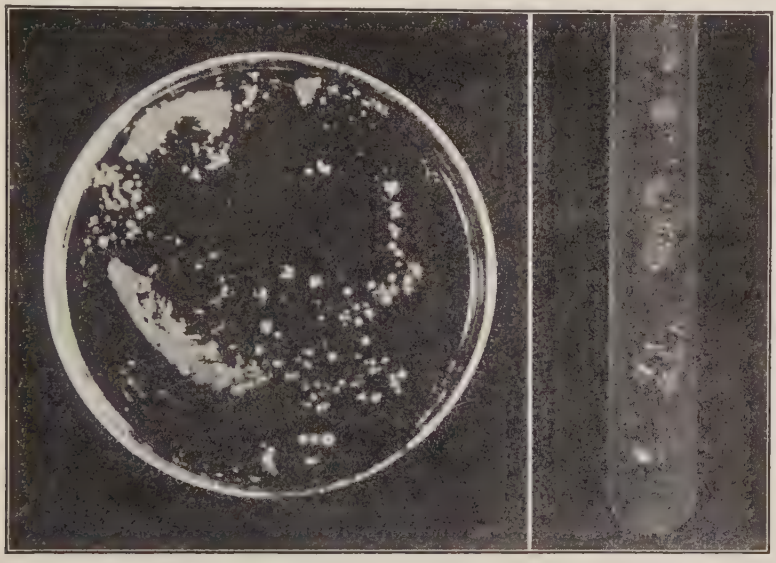


FIG. 200.—The d'Herelle phenomenon, plate culture, and agar slant, colonies partly dissolved out by the lytic principle; on the plate, solution almost complete. (*Photo from Kral Institute.*)

FIG. 201.—Action of bacteriophage on smear culture. Numbers outside the squares indicate number of bacteria in millions per cubic centimeter of emulsion taken; numbers inside squares indicate number of clearings from lytic action. The number of clearings is independent of number of bacteria used. (*From Asheshov.*)

solved," only small islands of undissolved and resistant bacteria remaining scattered over the original area (Fig. 201).

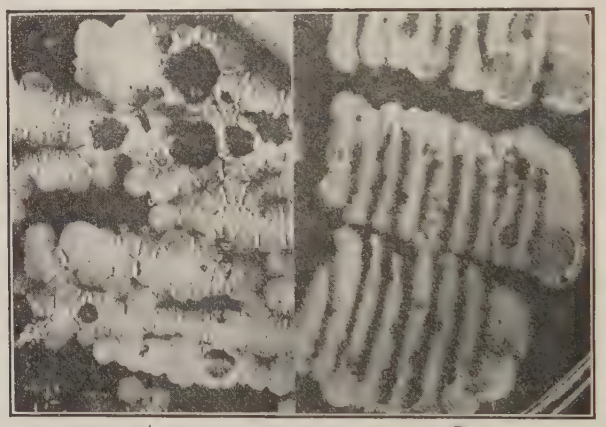
Varieties of Organisms Affected.—The bacteriophage principle was discovered in intestinal organisms, but d'Herelle[11] lists at least 24 species

in which it has been shown to occur, and others have since been reported. Among the organisms included are those of diptheria, those from the nodules of the legumes, *B. subtilis*, as well as a long series of intestinal species such as *B. dysenteriæ*, *B. typhosus*, *B. paratyphosus*, *B. coli*, and *Vibrio choleræ*. Their discoverer believes that every organism will be found to have a bacteriophage principle.

The strains of bacteriophages isolated by d'Herelle[11] (pp. 365 to 369) were considered by him to be as distinct, and as permanent, as bacterial species. While the strains may be modified temporarily or permanently by their growth conditions, bacteria are subject to about the same fluctuations.

OTHER LYTIC ACTION IN BACTERIA

A number of organisms show, in cultures, a clearing up and dissolution which resembles, in many ways, the process described by d'Herelle. These phenomena seem to be especially easy to obtain on some strains of



A B

FIG. 202.—*A*, lytic areas in Monilia agar culture; *B*, lytic areas in *B. pyocyaneus*. (*From Sonnenschein.*)

B. pyocyaneus (Fig. 202) where they have been studied by Hadley.[6] The agar cultures undergo a degradation in spots which spread and dissolve the bacteria, leaving a transparent, glassy growth. An examination of material from such a spot shows small granules which stain readily; these bodies probably being the certain remnants of the bacterial cells. Hadley has called this lytic reaction of *B. pyocyaneus* "transmissible autolysis," a term which simply means that the cells break up internally, due to the influence of a body which may be carried from other cells.

The growth of colonies affected by the lytic principle is much more rapid than that of the normal types, so Hadley suggests that the first effect of this destructive agent is such an intense stimulation of growth

and of disintegrative activity that an "explosion" occurs and the cells undergo lysis. As will be noted later, similar growth stimulation occurs in the cells affected by d'Herelle's bacteriophage.

d'Herelle's objection to this lytic principle as a bacteriophage is that cultures of susceptible *B. pyocyaneus* strains will, at times, break up without the addition of any of the filtrate containing it. Hadley's suggestion is that certain cells may carry this lytic principle in a latent condition within themselves, and that it becomes active if the physiological conditions become favorable. The balance is very slight in some instances and only a very little more tension sets it in action.

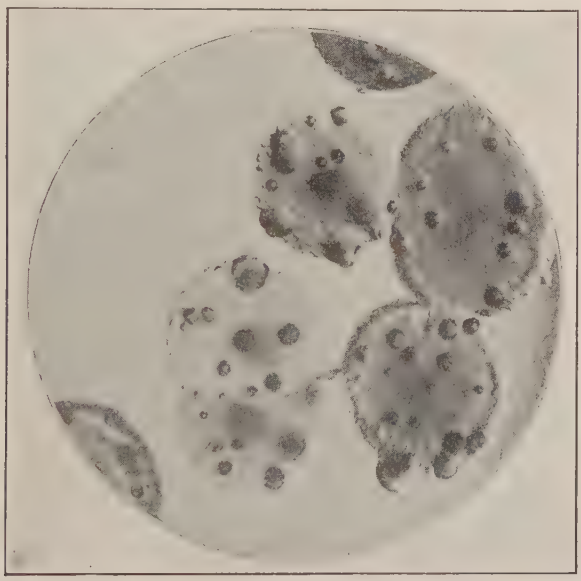


FIG. 203.—Secondary colony formation in *Vibrio choleræ*. (*From Eisenberg.*)

The lytic action in these *B. pyocyaneus* cultures represents an interesting phase of bacterial dissociation, regardless of its relationship to the d'Herelle phenomenon, although probably related in some manner to it. Similar lysis and the formation of secondary colonies have been studied in other organisms, such as the *Vibrio choleræ* (Fig. 203).

The Action of the Bacteriophage on the Cells.—The bacteriophage, according to its discoverer, is an "autonomous ultramicroscope corpuscle" which can be seen entering the body of the bacteria and multiplying there. The bacteria begin to swell after the bacteriophage has entered, possibly due to the multiplication of the parasites inside the cells. The affected cells, after becoming larger, but with a distinct wall, will suddenly disappear in a few seconds, and leave only a mass of amorphous debris.[12] This swelling and disappearance has been followed on indi-

vidual organisms by the use of a cineomatographic record. The ectoplasm of the swollen cells seems to be intact but the contents seem to stain irregularly. The dissolution process (according to Bronfenbrenner[12]) is probably a hydrolytic one with increased osmotic pressure inside the cell until it ruptures, and the products of the intracellular contents diffuse out into the medium, and are no longer recognizable.

The action may be divided into two stages, as already noted: (1) an early stage when cell growth is stimulated and (2) the swelling and bursting of the cells.

THEORIES REGARDING THE NATURE OF THE BACTERIOPHAGE

The nature of the substance which appears in the filtrate is still a matter of dispute. Some of the properties displayed by the bacteriophage resemble those associated with bacteria: the destruction by chemicals (by alcohol in 6 to 48 hours) and by enzymes. Other characteristics would seem to point to its resemblance to a ferment: its resistance to chloroform while bacteria are, in general, not active under the influence of chloroform, while enzymes are not affected. Only a few of the disputed aspects of the bacteriophage problem can be included; others are to be found in d'Herelle's book[11] and in Hadley's monograph.[8]

The chief theories may be grouped as follows:

1. *Theory of d'Herelle, Virus Theory.*—(The bacteriophage is a foreign body which attacks the bacteria; it represents a single species (unicity) but may have various strains; is a filterable virus.)

2. *Theory of Bordet, Enzyme Theory.*—(The bacteriophage is an enzyme-like body, internal, and metabolic.) Lysis is the result of a "nutritive vitiation."

3. *Theory of Hadley, Homogamic Theory.*—(The bacteriophage is connected with the dissociation in bacterial cells; is a normal cell function released by proper external stimuli; is of significance in bacterial reproduction.)

THE THEORY OF D'HERELLE

d'Herelle continues to hold the doctrine that the bacteriophage is living, a parasite, not a part of the bacterium, autonomous, and in itself, an entity. He attempts to prove it by the following experiment:

If each of 10 tubes, containing 10 centimeters of a culture of the Shiga bacillus, but in varying numbers, are inoculated with the *same amount* of a strong filtrate of the bacteriophage, it will be found that, if $\frac{1}{50}$ centimeter is taken from each tube and stroked on agar slants, each slant will show the same number of sterile spots induced by the bacteriophage. The number of these spots is practically the same on all of the cultures. But, if, on the other hand, 10 tubes of the Shiga bacillus is taken, all

containing the same numbers of bacteria, and to them are added *varying amounts* of bacteriophage filtrate, the number of spots will vary and will agree fairly well with the amount of bacteriophage that has been added (Fig. 201). These experiments would indicate that the number of dissolved spots is dependent *not* on the *number* of bacteria introduced but on the *amount* of the bacteriophage. d'Herelle is convinced from experiments of this sort that he is dealing with a definite element which produces the spots, and that this ultramicrobe or "protobe," acts as a parasite on the bacteria in the cultures.

This experiment has been repeated and confirmed; a given dilution of bacteriophage will produce an approximately equal number of lytic areas regardless of the number of bacteria affected. The results also show another property of the bacteriophage; it does not increase in numbers in a filtrate. While autonomous, it cannot reproduce itself unless in contact with bacteria; it is a product of the lytic action of the bacterial cells.

The multiplication of the bacteriophage corpuscles occurs only in the presence of susceptible bacteria. The following summary by d'Herelle" (p. 116) shows the time required:

"After 30 minutes of contact at 37°C. the bacteriophage have almost entirely disappeared from the liquid.

"After 60 minutes, the situation is the same.

"After 90 minutes the corpuscles have *suddenly* reappeared in the liquid, and this number is 18 times greater than was that of the inoculated corpuscles. In other words, each inoculated corpuscle has yielded 18."

The Size of the Lytic Areas (Plaques).—The size of the areas in which the bacteria are dissolved varies with the strain of bacteriophage used; virulent strains, according to d'Herelle, inducing the larger ones. Other investigators (Gratia) have laid emphasis on the resistance of the bacteria in contact with the bacteriophage, the type of organism being as important as the strain of bacteriophage. Large and small plaques (dissolution areas) appear, as well as all intermediate sizes also.

Time in the Life of the Bacterial Cell at Which the Bacteriophage Appears.—According to Bronfenbrenner, the bacteria do not have to undergo dissolution in order for the bacteriophage corpuscles to increase in numbers; the infected bacteria giving them off in quantity during the early stages before the lytic action can be seen under the microscope. d'Herelle[11](p.122) claims that the increase in corpuscle numbers occurs as the bacteria burst in the old cultures. The experimental evidence cannot be given in detail, but an important theoretical principle is involved. If the corpuscles are secreted from the bacteria soon after infection, they are comparable to enzymes or toxins; if largely from the dissolution of the organisms, they are parts of the bacterial cell from which they have been ejected.

THE THEORY OF BORDET

Bordet[1] interprets the action of the bacteriophage as a metabolic and inheritable disturbance of some of the organisms, a *nutritive vitiation*. Only a part of those which appear in a culture are affected in this manner and go to pieces under certain conditions by an *autolysis* (auto = self, lysis = digestion) of the bacterial cells. Bordet would substitute for d'Herelle's bacteriophagy, the term, "transmissible bacterial autolysis."

In every bacterial culture an equilibrium exists between assimilation and metabolism; if this balance is destroyed, a "nutritive vitiation" results which may result in a pathological condition in the cells. Any culture is susceptible to this lytic action, but some are more resistant than others; the equilibrium between them and their environment is more stable.

THE THEORY OF HADLEY

Hadley[7,8] sees in the bacteriophagic action not a parasite entering the bacteria from without, nor a metabolic condition due to environment, but a transformation of the organized bacterial cell as a part of the life cycle which it undergoes When a predetermined phase in its life history (induced partly by the environment, but fundamental to the protoplasm) is attained, the culture may undergo lysis. This lysis may be so complete that not even granules from the cells can be found, or it may leave densely staining bodies undissolved.

This autolysis of bacterial bodies, as part of the dissociations which appear in cultures at times, leads to new phases in the life history of the organisms. An organism may pass through a growth of the usual type but then become transformed by these "lytic" principles into a second type, or phase, which may be radically different from that from which it arose. These dissociations are the result of an internal and intrinsic condition which is not to be regarded as a disease but as an entirely normal part of the life cycle. As d'Herelle points out (p. 316), the autolytic (metabolic or normal) view is largely accepted by Anglo-Saxon investigators; the parasitic by the Latins.

OTHER VIEWPOINTS AS TO THE NATURE OF BACTERIOPHAGE

Bacterial Fragments May Be Associated with an Enzyme.—The corpuscles of d'Herelle are believed by some other investigators to be not the bacteriophage itself, but only a colloidal base to which the active agent is attached in somewhat the same manner as enzymes are composed of two parts, the active agent and its colloidal base.

Otto, Munter, and Winkler[14] hold that the bacteriophage arises as an active principle from the bacteria themselves, it is the fermentation unit, the smallest part into which the living bacteria, as we know them, can be broken. Bacteria are composites made up of units with a variety of functions, but which may be separated, each unit functioning in its charac-

teristic manner independent of the other units. Some of these units (of the enzymatic type) may become parasitic and result in the disintegration of the cells which they enter.

The Bacteriophage May Be an Autolytic Enzyme.—The bacteria are assumed to contain certain enzymes which cause them to pass from one phase to another in their life cycle. Gildemeister[5] is of the opinion that these digesting principles represent one phase in the life history of the bacteria; they are an example of the lability of the bacteria in that the form of the organism may change. The bacteriophage is only one phase in the variability of organisms; the cells may even disintegrate into a more or less formless mass which still possesses many of the properties of the organized individuals, but whose particles may act as parasites on the bacteria from which they arose.

Bacteriophage as a Possible Cause of Variation and Mutation in the Bacteria.—The causes of this series of internal changes which may lead to new species are also probably very complex, and the explanations offered vary. D'Herelle[11] (p. 217) would attribute the formation of secondary colonies in old cultures to the action of bacteriophage on the more susceptible individuals, leaving a few more resistant and slightly different members which give rise to a new race, somewhat in the same manner as an epidemic, such as the plague, fatal to most human beings, might wipe out all except a few favored by nature. The new, and more resistant, race is somewhat different biologically from the normal strain from which it arose, one phase being the marked vitality of these resistant types. The new resistant race might be considered to be a mutant. The factor which induces the mutation in this case is an external one, although it acts internally.

Hadley[7] would see in such an epidemic, not an *external* factor at work but an *internal* one, a lytic principle which breaks down and transforms nearly all the individuals but leaves a modified type, which is not seriously affected, to continue the species. This internal factor, carried by all the organisms in a latent condition, only comes into action when the external conditions are such as to set it in operation. The organisms in which it is weak or lacking remain to start a new race, a mutant, which is also different in characters other than those of resistance to it.

Such a theory strongly suggests the orthogenesis of Eimer; progress is made along definite lines, conditioned by the internal constitution of the plant or animal, this constitution acting in the surroundings in which it must live. Each step in advance is dependent on the previous ones, as expressed in the protoplasmic substance at that particular time.

BACTERIOPHAGY AND LIFE CYCLES

The entire problem of dissociation is too complex to discuss in detail, but the possible relationship of such autolytic action to the life cycles

discovered in some of the bacteria by Löhnis and Smith[13] (following chapter) must be noted. The morphological evidence of such a disintegration in many types of organisms may have its physiological basis in bacteriophage action. Hadley would not make the same limitations to bacteriophagy as d'Herelle, but would include under the term "dissociation phenomena" the internal "dissolution," or transformation, of bacterial cells, regardless of the sizes of the products, *i.e.* spherical bodies, small granules, or an amorphous mass.

THE IMPORTANCE OF THE BACTERIOPHAGE IN BACTERIOLOGY AND MEDICINE

The importance of bacteriophagy in bacteriology, both theoretical and applied, cannot be easily overestimated. Some of its possible effects have already been noted: An old strain of organisms may become almost unrecognizable after it has been affected by lytic action; new strains may arise under its influence; old strains may change many of their biochemical reactions (such as chromogenesis); fermentive reactions may be altered; virulence may be weakened; and toxicity may be lost.

Organisms usually considered to be non-filterable may be broken up so that they will pass even fine-pored filters in the same manner as filterable viruses, but regain their original form and properties. For example, Eichhoff[2] claims to have filtered the typhoid organism, apparently in a disintegration stage, and Fejgin[4] to have obtained a filterable virus from the typhoid organism which will produce the disease in guinea pigs, the original culture having been treated with a bacteriophage; the original rod-like culture, after treatment with a bacteriophage, having been transformed into minute cocci.

THE BACTERIOPHAGE IN MEDICINE

In medicine the lytic principle has been used as a serum:

A. As a prophylactic agent (for the prevention)
   1. Of dysentery.
   2. Of typhoid.

B. As a therapeutic agent (for the treatment)
   1. Of dysentery.
   2. Of typhoid.
   3. Of suppurative infections (streptococcus and staphylococcus).
   4. Of plague.

d'Herelle's suggestion, that the beginning of some of the communicable diseases, such as typhoid and dysentery, is due to the lack in the body of a strain of bacteriophage which could attack the organisms successfully, or that the strain of invaders became resistant, has been supported by some of the evidence obtained. Others find in the introduction of the bacteriophage only a modification of the normal course of the disease.

The majority of the effects which have been obtained are favorable, although many negative results are also recorded.[8] While the usual practice in such treatments is to inoculate a bacteriophage of the same type as the bacteria which it is to dissolve (since the bacteriophage seems to have virulent and avirulent strains as do the bacteria, the virulence being indicated by the size of the lytic plagues which appear in cultures on solid media) a strain should be selected which is derived from the bacteria upon which it is to act, and should produce an area of maximum size.

The bacteriophage, while it has not met all the sanguine hopes of some investigators for the treatment and cure of a number of important bacterial diseases, especially those of the alimentery tract, is a very hopeful agent.

THE BACTERIOPHAGE AS A REVOLUTIONARY ELEMENT

Many problems regarding the bacteriophage and its action remain to be solved, but no field of biological research work, since the pioneer days of Cohn, Koch, and Pasteur, has been so actively cultivated, or has opened so many lines of thought; d'Herelle's book[11] and Hadley's monograph[8] together contain over a thousand titles of papers on the subject, mostly published since 1916. The theories which have been advanced are fundamental and revolutionary. The results which have been obtained, have been, to a large extent, ignored by the old-school bacteriologists, a condition of indifference which can no longer be maintained, since the modern school of investigation has developed a technic which is a distinct advance.

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CHAPTER XLIII

MODERN ATTACKS ON THE CONSTANCY OF SPECIES

(Continued)

LIFE CYCLES IN THE BACTERIA

Morphological Changes in the Size of the Organisms Occur at Different Periods of Growth in the Same Culture.—Manuals of systematic bacteriology give measurements supposed to represent the limits within which an organism varies, these figures being taken from observations on fairly young cultures, *i.e.* 24 hours old. Evidence has been accumulating in recent years that such measurements are only accurate for one particular age of culture; size, grouping, and cell contents change from hour to hour. The most easily recognized symptom of change is in the size of the cells.

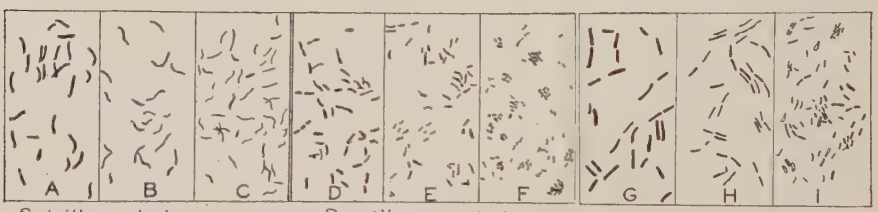

FIG. 204.—Variations in cultures of different ages. *Sp. cholerae*, 2-, 12-, 24-hour old cultures; *B. prodigiosus*, 2, 4, and 12 hours; *B. subtilis*, 6, 9, and 18 hours. (*After Clark and Ruehl.*)

Age and Size in Bacteria.—Clark[3] and Clark and Ruehl[4] measured 70 strains of organisms and found that, with the exception of the members of the diphtheria group and, possibly, those of the *B. mallei* group, the individuals were very much longer in the 3 to 4 hour old cultures than in those 24 hours old (Fig. 204). The period of maximum size coincides with the time during which the organism is dividing most rapidly. Similar observations made by Henrici,[11,12,13] on a series of organisms of which *B. megatherium* may be taken as typical (Figs. 205 and 206), show individuals which have just been transferred to be small and granular but, in succeeding divisions, the cells become larger and the granules seem almost entirely to disappear; the small, granular phase being coincident with the "lag" phase in cultures. Henrici divides the lag phase into two parts: an initial dormant phase and a succeeding phase of accelerating

growth. The interesting suggestion has been made that the individual bacteria from an old culture are in an incipient spore state. The organism must "germinate" before it is normal as to size or rapidity of growth, and this stage is the lag phase. Another interesting physiological explanation of the lag period is that it is due to a depletion of the CO_2 in the

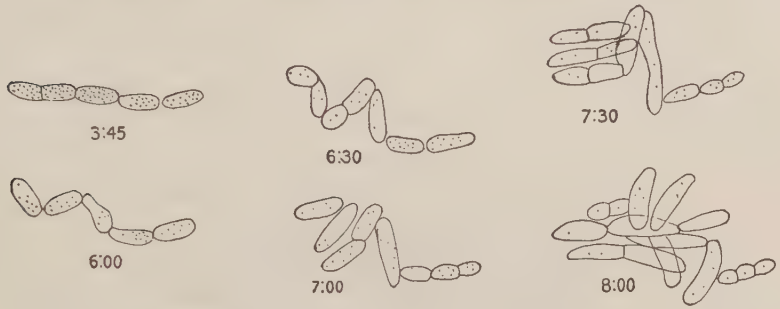

FIG. 205.—Size and granulation of *B. megatherium* at successive intervals of time, semi-diagrammatic. (*After Henrici.*)

cells; the cells, in order to absorb and grow at a maximum, require a certain amount of carbon dioxide inside them. When transferred from an old culture to a new one, the depletion continues for some time.[17,26]

Whatever may be the true explanation for the lag period and for the variations in length, young organisms are always found in the cultures

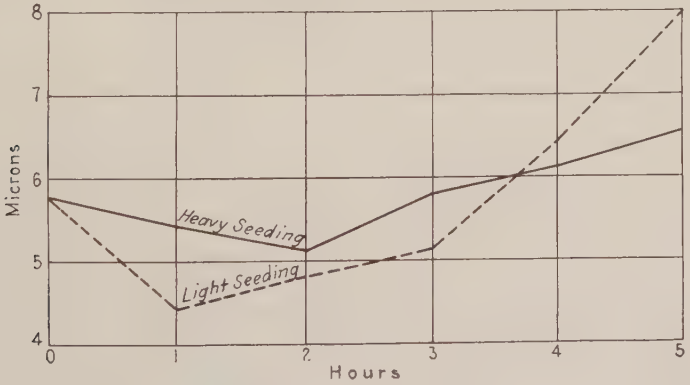

FIG. 206.—Effect of age and seeding of a culture on length of cells of *B. megatherium*. (*After Henrici.*)

where cell division is at a maximum and, as it falls off, the size diminishes. The old age which comes to the individuals in a culture manifests itself in a decrease in size, due to lack of food in competition in a dense mass of individuals, to a decrease of certain gases, or to an increase in the concentration, inside and outside the cell, of toxic excretion prod-

ucts. Possibly the combination of all these factors results in the senile individuals.

Involution and Degeneration Types in Old Cultures.—In some species, such as the legume nodule organism, *B. radicicola*, the interesting fact has been observed that the active bacteria in the nodules assume irregular shapes, while in cultures they are more of the "normal" rods. Later observations have discovered a succession of shapes in old cultures of this organism (page 229).

Similar irregular shapes are to be usually found in old cultures of almost any species and, being considered degenerate, have been called "involution" forms. Some organisms have been known to go, at times,

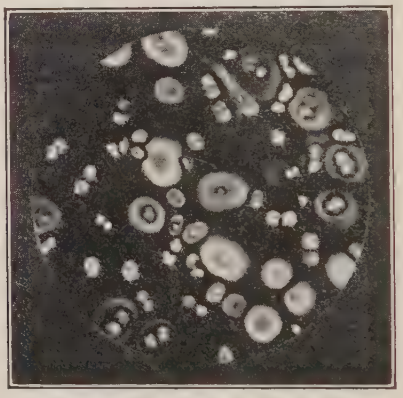

FIG. 207.—Azotobacter cells breaking into smaller bodies inside the capsules, × 1,000. (*Photograph by Prof. D. B. Jones.*)

into a branching myceloid stage and, in the case of the tuberculosis organism, this appearance had led to the new name of *Mycobacterium*. In bacteria in which such a stage occurs, it has simply been ignored or ascribed to degeneration induced by the crowded surroundings and accumulated excretion products of old colonies.

The breaking up in old cultures of the rod-like forms of Azotobacter internally into spore-containing and coccus colonies had been noted by Löhnis and Westermann,[20] but Jones,[15,16] in a series of convincing photographs (Fig. 207), conclusively demonstrated the succession of very marked changes which cells of Azotobacter undergo. These phases could be followed from one into the succeeding one and proved that the conception of fixity of form was incorrect for Azotobacter cultures.

ARRANGEMENT INTO LIFE CYCLES

The careful and detailed study of these stages in Azotobacter and in 41 other varieties for comparison, by Löhnis and Smith,[21] led them to the theory that these organisms and, perhaps, many others, go through

a definite life cycle, the various stages being marked by observable morphological changes; the conclusions being confirmed by Jones,[16] with a few exceptions. The old pleomorphists believed that one organism could be transformed into another of a very different form, a coccus into a rod, for example, if cultural or environmental conditions are altered. While the changes, as observed by these later investigators, however, do not imply any such transformations, they been called the "neopleomorphists." The rod shaped organism in pure culture may break up

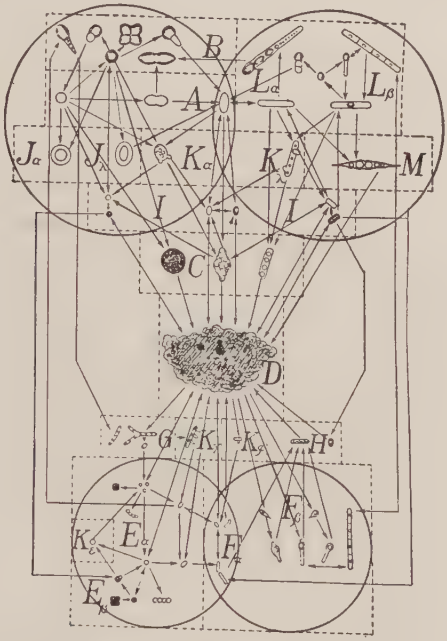

FIG. 208.—The life cycle of *B. azotobacter*. (*From Löhnis and Smith.*)

into cocci, but it is always the same species presenting itself under a new morphological aspect.

The accompanying diagram (Fig. 208), together with the series of photomicrographs (Fig. 209), will give some idea of the succession of forms which may appear in a culture of Azotobacter. The essentials are *A*, *D*, *G*, and *F*. The ordinary forms, such as are found in young cultures, are the spherical or slightly elongated types at *A*. These may go through various modifications and break up into small gonidia, which in turn grow to normal forms or to some related form.

At some time in the life of the culture, the cells pass into an amorphous state in which the individual organisms cannot be distinguished. In this amorphous mass, regenerative granules appear which may give rise to the normal organism or to a coccoid or other form. The changes in mor-

phology may occur, therefore, either in the upward growth of the regenerative bodies or in the development of the gonidia without going through the symplastic (amorphous) stage.

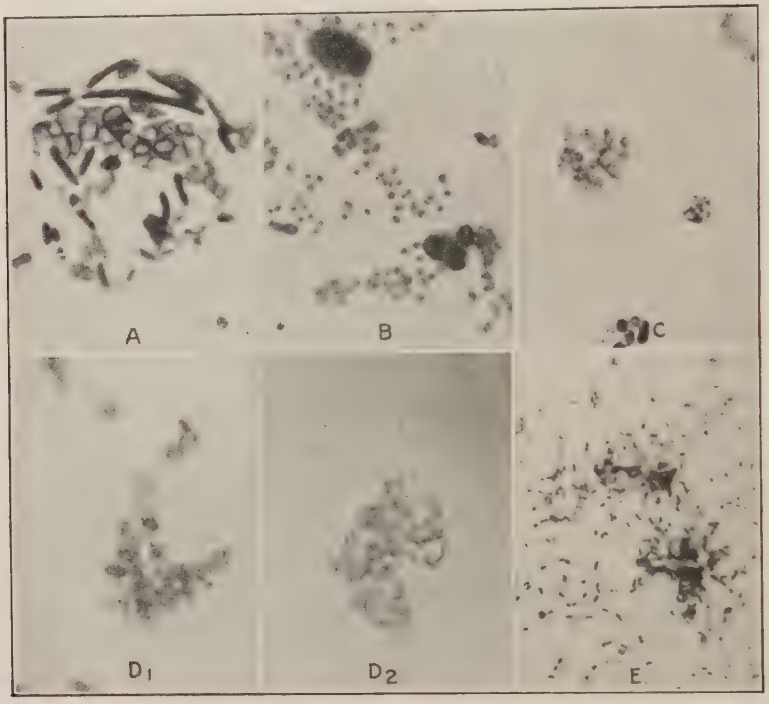

FIG. 209.—Phases in life cycle of Azotobacter; stages numbered as on diagram. This series of pictures is a striking illustration of the variation in appearance of the same organism in the same culture. (*Photographs from Smith.*)

THE AMORPHOUS OR SYMPLASTIC STAGE

The amorphous or symplastic stage has been the most difficult one for bacteriologists to accept; the changes in form and size in cultures have been long observed, in fact, since the early days when pure strains could be obtained; but the explanation offered, that this process is not a real degeneration but only a stage in the life-cycle, is new. This amorphous condition of bacteria in old cultures had already been noticed by Bastian[2] who regarded it as a stage in the evolution of the bacteria from the organic and inorganic elements which compose them. Bastian was interested in the origin of life and considered that he had found, in these amorphous appearances, the connecting link between the living and the non-living. He was unable to avoid some of the objections raised by Pasteur and Lister and his observations have fallen into disrepute but, while many of his views regarding abiogenesis are undoubtedly without foundation, the amorphous masses which he observed have to

be explained in some way. The organisms in these masses, if the Löhnis-Smith theory is correct, could be destroyed by heat and chemicals in much the same manner as the usual types seen in the ordinary bacteriological cultures.

Löhnis and Smith offer no explanation for the life cycle which they have described, but the internal lytic principle may be the physiological basis for the morphological fragmentation which they figure. The fragments may, in this case, be large enough to survive and continue growth, while in lytic dissociation the bits of protoplasm are too small to continue, unless in association with another cell.

Symplasm and Filtration.—If such a regeneration of bacteria can occur from an amorphous condition, and if bacteria at some time form very small microgonidia, many phenomena which have hitherto been rather difficult of explanation can be cleared up. The passage of bacteria through very fine filters, especially after they have been in use for some time, needs explanation. Eichhoff,[7] for example, claimed to be able to suspend typhoid organisms in water, pass them through a filter, and, from the filtrate, cultivate them and see them under the microscope. Such claims need confirmation but the passage of pathogenic organisms through body membranes, such as those of the alimentary tract, and the variety of forms under which some of the pathogenic types appear, could all be explained if we assume a life cycle in which not only the form of the organism changes, but in which it may be lost entirely for a time. In the soil, the bacteria may undergo such a series of changes, performing various functions while they are at various phases of development.

The Eriksson Mycoplasm Theory.—In this connection, attention must be called to the very similar views which one of the most noted students of the parasitic fungi, Eriksson[9] has expressed with regard to the rusts. These highly organized fungi, the grain rusts, are able (according to Eriksson) to lose their form entirely at one stage in their development and pass, as an amorphous mass known as "mycoplasm," through cell walls and plasma membranes to find a resting place inside the host cells of the wheat grain where they remain in a quiescent condition during the winter. They start up again in the spring, creep back through the cell walls in the plasmoid condition and resume their shape, wall, and activities in the intercellular spaces of the young wheat seedling. The Eriksson theories have been rejected by the majority of mycologists, but they are still somewhat at a loss to explain the overwintering of the grain rust when no visible evidence of it can be seen on the grain itself nor on its alternate host plants which in places may harbor it. The sudden epidemics of plant diseases which appear when the weather conditions are just right are impossible of explanation unless we assume that many of the fungi spend a large part of their existence in the soil in a purely saprophytic manner; an admission that is as repugnant to the extreme

follower of Koch as that the organisms are pleomorphic and have a complicated life cycle, through which they may pass, and in which they may lose their "constant" form.

STABILIZING STAGES IN THE LIFE CYCLE

A culture usually contains a number of different stages, but one or two forms are predominant at any time. In these mixed growths, the various components can be separated out by plating, the colonies which appear on the plates usually being composed of the same form of organism as on the original tube of Azotobacter,[22] although they occasionally revert to type. The strains isolated in this manner are very persistent and last for years.

This method seems to give just as good results as does the isolation of the various phases by picking up some of the cells and transferring them to new media by means of some mechanical device, such as the Barber

FIG. 210.—Life cycle of a diptheroid bacterium: *A*, usual form, long slender rods; *B*, fungoid stage when branching may occur; *C*, the giant coccoids which arise from the branching type. (*After Mellon.*)

apparatus. The latter method has been used, however, by some investigators who claim that it is superior to plating out. Some of the phases in a diphtheroid organism isolated by this technic by Mellon[25] are to be seen in Fig. 210. The ordinary type of long slender rods is shown in *A*, the myceloid forms at *B*, and the giant coccoids at *C*. All of these phases appear in a single culture which he used for his experimental work. Inside the giant coccoid forms, at times, appear granules which may represent still another phase in the life cycle. The dominant phase is dependent on the culture, since the cycle is followed in each typical culture and some individuals of all stages may be found at almost any time in stained slides.

THE LIFE CYCLE THEORY DESTROYS THE OLD MORPHOLOGY OF THE BACTERIA

The revolutionary character of the theory of life cycles has made it slow of acceptance by many bacteriologists, since it wipes out many of the land marks of morphology, as established in the past 40 years. The

generic differences which separate cocci from bacteria are broken down; for one may merge into the other, in the course of the life cycle; on the other hand, bacteria which are morphologically different may be the same species. The whole life cycle in a culture will have to be studied before two species can be said to be the same.

The validity of the theory can be determined only by widespread corroboration by workers in microbiology but it has aroused widespread interest in almost every country. Mellon,[23,24,25] in a series of papers on the diphtheroid organisms, Almquist,[1] on the typhoid organisms, Cunningham, and Jenkins,[5] on *Bacillus amylobacter*, Lochhead,[18] on *B. Alvei*, Gibson,[10] on *B. radicicola*, and David,[6] on a vibrio, have been among the investigators who have found life-cycle phases. On the other hand, many bacteriologists are still unconvinced that any such phases occur as a normal part of the life cycle; the granular appearance, breaking up of cells, budding, etc., they interpret, as did the older school, to be degeneration and aging phenomena,[27] with, however, some indications that these particles may survive and reproduce the organisms.

CYCLOGENY IN THE BACTERIA

A somewhat different attack on the unity of the morphology of species was made about the same time as the Löhnis and Smith contributions by articles on the appearance of reproductive processes, other than the simple fission, by Hort[14] in England, working on the typhoid bacillus, and by Enderlein[8] in Germany, working on a series of organisms. The elaborate life changes in cultures, which have been worked out by these two investigators in entirely independent laboratories, have been partly confirmed by other students, but much still remains to be done to determine as to how far they are correct in some of their interpretations.

The essential of the new interpretations and observations by these workers is that not only stages of *endosporulation* occur but also *conjugation* in the species observed. The endosporulation may be similar to that described in Azotobacter, but the conjugation is quite a different phase. The conjugation gives rise to larger bodies, known as "zygospores," which are sexually produced. One phase in the bacterial cycle would be a true sexual one.

The whole problem is considered by these authors from a cytological standpoint with very little reference to the work that has been done on the breaking up of the bacteria in cultures. Enderlein has originated a new and somewhat complicated series of names to indicate the different stages in the life cycle, these names being so unlike any of the old terminology that they have not yet been accepted by bacteriologists. Changes which appear to be cyclic have been taken up extensively by

Mellon[25] on diphtheroid organisms and by Almquist[1] on the typhoid organism.[17]

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CHAPTER XLIV

THE PROBLEM OF THE CLASSIFICATION OF THE BACTERIA

The systematist would say the object of all study of form and physiology was to establish the relationship of organisms to each other. The thread of relationship, leading through the jungle of isolated facts discovered by the students of physiology and morphology, will, if followed, lead to an inner elevated position where individuals and individual phenomena which have been passed can all be seen in their proper relations. If such an elevated outlook in microbiology exists, no one yet seems to have attained it; bacteriologists are still groping in the jungle.

The Relationship of the Bacteria to Other Plants and Animals.—The bacteria are closely related, according to almost all students of the group, to the Cyanophyceæ. The method of reproduction, and particularly the cell structure with its absence of a clearly defined nucleus, are responsible for this common position in the plant kingdom. The presence or absence of green coloring matter is of secondary importance.

On the other hand, the presence of flagella seems to relate bacteria to the flagellate protozoa, such as *Spumella vulgaris*, as was suggested by de Bary,[1] since this uniflagellate protozoan forms single endospores or cysts. De Bary emphasized the fact, however, that the cyst formation might only be an analogous appearance of no homologous importance. The same author calls attention to a flagellate stage in the slime molds, and to the occasional production of flagellate spores in many green algæ and in the lower fungi.

At the present time, the exact relationship of the groups forming the *Protista*, the primitive plants and animals, cannot be determined, but the fungi, flagellate protozoa, algæ, Cyanophyceæ, and the bacteria must be assumed to have developed along somewhat parallel lines from some hypothetical ancestor. The flagellates, some of the fungi, algæ, and bacteria have retained or further developed the organs of motility, while the Cyanophyceæ and the bacteria have retained the simple, primitive, undifferentiated cell structure in which the nuclear material is not separated from the cytoplasm.

The Relationship of the Bacteria to Each Other.—Systematists dispute less over the question of the limitations of the various genera than they do over the vexed problem of species, since generic characters are usually well defined but, in the bacteria, the limitations, both of

genera and species, are uncertain, since we are at a loss to define the term "species."

THE PROBLEM OF SPECIES

In plants and animals of large size, with characters well marked and the individuals large enough to examine in detail, (both externally and in their finer structure, such as the chromosome numbers) the answers to the question, What is a species? are diverse; so it hardly can be expected that among forms which are so minute that they can be examined only with the highest powers of the best lens of the compound microscope, that there will be any probability of any conclusions of very great value from the study of the morphology of the microorganisms themselves, unless combined in some way with other characteristics. The morphological characters are so constant in the higher groups that we instinctively fall back on them as the basis of any classification; so the earliest students of the bacteria, in attempting to arrange and name them systematically, made *form of the organism* the basis for the nomenclature in almost all cases, using any physiological characteristics as secondary means of determination. The names which are in use today arose during the early part of the last century and were carried over by the great German student of the bacteria of the middle and latter part of the century, Ferdinand Cohn,[7] who laid down the principles upon which the later bacteriologists have built. This classification, largely a morphological one, was not entirely new even in Cohn's time, since it goes back still further to Ehrenberg for many names, and the basic thought of spherical, rod-shaped, and spiral groups. Cohn, however, greatly amplified it and worked into the scheme many of the discoveries which he and other investigators had added since Ehrenberg's publication in 1838. The inadequacy of morphological classification was evident, however, and could be extended only to cover the genera; Cohn's species being founded principally on physiological characters, such as color production, fermentation of urea, or the production of a typical disease, such as anthrax or diphtheria. He was mistaken on some of these characters, such as those of his supposed micrococcus of diphtheria, so that many of his species are not valid.

His studies were so basic, however, that the American Society of Bacteriologists once dated their beginning of nomenclature from their publication in 1872. This action was reversed 10 years later, and the publication of Zopf's "Die Spaltpilze" in 1885 was made the beginning for names. American bacteriologists now accept any name which can be associated with an organism since 1753, in this way attempting to do away with national jealousies.[3]

PHYSIOLOGICAL CHARACTERS BECAME NECESSARY FOR IDENTIFICATION

As soon as the discovery of numerous organisms of the spherical, rod, or spiral shape, apparently quite different in their growth and effects on

various culture media, made it evident that, although under the microscope apparently identical, they were distinct species, bacteriologists made lists of the differences in culture, for use as tests, by which an observer could determine the minutest points in morphology and physiology for comparison with those discovered on organisms which had already been named. These characteristics, listed by Dolley,[8] the result of the work of bacteriologists, such as Koch, Pasteur, Zopf, and others, have not been materially changed since that time; details have been added as slightly improved methods have been discovered:

"A thorough study of any peculiar bacteria must answer the following questions:

1. Shape, size, color, and details of structure, *e.g.* flagella, peculiar envelope, etc.? Character and speed of movements?

2. Character of natural habitat? Artificial media best adapted to growth and reproduction? Stages of development passed through? Formation of zoogloea, spores, filaments, rods, cocci, swarms? Conditions under which such formation occurs? Character of colonies formed in firm culture media?

3. Capability of producing fermentation? putrefaction? character of decomposition products, volatile and other, formed in various nourishing media?

4. Behavior towards oxygen at normal and altered pressures? Behavior towards other gases?

5. Effects of various temperatures on movement, germination, etc.?

6. Behavior in relation to light (phototonic properties)? Behavior towards electricity?

7. Behavior towards antiseptics and poisons?

8. Are the forms under investigation found in a diseased organ or tissue? What is the effect of inoculating animals of different orders and species with pure cultures? If virulent, can the virulence be attenuated by exposure to air, to antiseptics, to heat, or by repeated 'fractional' cultures? Under what condition? Does the inoculation of attenuated germs have a cumulative effect if repeated at short intervals? Does one inoculation give immunity towards a second made with virulent microbes?"

The above list of characters is essentially the same as that issued by the American Society of Bacteriologists, known as the "Society Chart." The characters have in some cases been elaborated, but in general the whole method is the same: the description of the organism, of the colonies, growth on slants of agar, growth on milk, gelatin, etc., the growth and gases formed in the fermentation, acid or alkali production, indol production, reduction of nitrates, etc., have all been much elaborated, but the basic principle is on exactly the same lines as those laid down by very early workers. Manual descriptions hardly ever agree in all details with any newly isolated strain, as can be shown in a diagrammatic manner (Fig. 211). A typical species of *B. coli* would show a maximum of each of the characters indicated but an isolation might show some of them typically, others less so, and, for some strains, the character would be almost or, possibly, entirely lacking. Still, this culture would be considered to be one of a member of the *B. coli* group. Two strains isolated from two different sources may be said to be the

same organism, if, grown under identical conditions on the same media, they both have the same characters, although differently accentuated.

Variability Causes Difficulties in Classification.—The difficulty with any system of classification of plants or animals lies primarily in the wide variations to be found within any group that may be established. The individual cannot vary, but any general description applied to two, three, or more individuals at once encounters difficulties, because no two individuals have exactly the same size, shape, or physiological characters. This variation may be illustrated from a morphological standpoint by the various lengths given for any one species of bacteria. These length measurements are usually made from supposedly pure cultures grown for 24 to 36 hours. The length of an organism such as *B. prodigiosus*, is from 0.5 to 1.0 micron in length; for *B. coli*, 1.0 to 2.0 microns; and for *B. subtilis*, 1.5 to 2.5 microns.[2] These measurements probably do not represent the extremes, for still longer or shorter individuals could be picked, or, as already pointed out, by using special, or unusual, culture media, the organism can be further modified, so as to be temporarily, at least, unrecognizable from a morphological description, and the age of the culture induces further changes.[24,27]

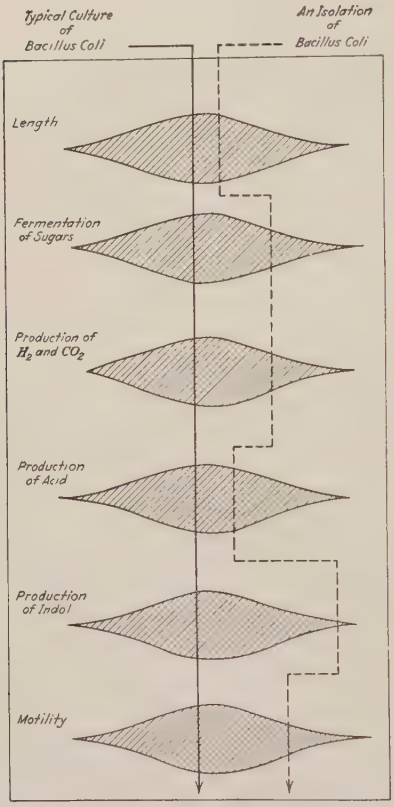

FIG. 211.—Comparison of single isolation of an organism with the possible variations of the species.

PHYSIOLOGICAL CLASSIFICATION

The unreliability of morphological characters led Orla-Jensen[38,39] to suggest that they be largely abandoned and physiological ones substituted. For example, all lactic-acid producing organisms would form a large group which might be subdivided on other physiological or morphological differences.

The morphological characters of microorganisms would have a very different rating by Orla-Jensen[38] than that given them by Cohn or Migula:

"Of the three principal morphological qualities of bacteria; the arrangement of the flagella, the shape of the cell, and the spore formation; the arrangement of the

flagella is of primary importance. The shape of the cell, on the other hand, is merely a generic character, and we may, within one and the same family, encounter sphere, rod, and screw forms, as is well known in the case of the red sulphur bacteria, and as we shall also find from an example among the lactic-acid bacteria. As all these bacteria appear immotile, and do not form spores, we have, in reality, no morphological indication whatever, to go upon when placing them in the bacterial system. On the other hand, their biological qualities are so pronounced, inasmuch as they require just as complicated nourishment as animals, that there can be no doubt as to their place, *viz.* among the order of *Peritrichinæ*."

The conclusions of Orla-Jensen of some years ago,[38] are further emphasized in his paper on lactic-acid organisms: "We find that all bacteria which are able to live exclusively upon inorganic nourishment, and derive their energy through simple oxygen processes, have the flagella terminally set, whereas those requiring more complex organic food, and producing the more typical fermentation processes, have the flagella distributed throughout the whole of the cell. On this basis, therefore, I have divided the bacteria into two orders: *Cephalotrichinæ* and *Peritrichinæ*."

The Orla-Jensen physiological classification would have, as shown in the latter quotation, its morphological basis; not on cell *shape* but on flagella *distribution*, since the latter seems to correlated with food requirements.

It is doubtful if any purely physiological classification would be any improvement over the older morphological one, since variation in physiological characters is as wide as those of form and size, as shown in the ability of various strains to ferment sugar, or to produce gas, indol, or other by-products. Elaborate studies on the physiological variation within a supposedly "good species" have been made on *B. carotovorus* by Jones, Harding, and Morse;[32] on the Coccaceæ by Winslow;[51,52] on *B. fluorescens* by Edson;[10] on *B. coli* by a number of investigators among which may be named Eisenberg;[11-16] on the old species of *Actinomyces chromogenus* by Krainsky,[35] Waksman and Curtis,[49] Lutman and Cunningham,[37] and Drechsler.[9] They all indicate that the meaning of "species" in bacteria is not clear and that any description that may be given is for practical purposes of identification and may represent no real relationship. Some one character is picked and on this a "species" is built. As examples of this practice, the characters of some of the organisms cited may be used; the fundamental characteristics of *B. carotovorus*, is its ability to rot cabbage and related plants; *A. chromogenus* by Lutman and Cunningham,[37] was considered a species largely because of the production of brown pigment in the medium; *B. coli* because of certain sugar-fermenting properties with the formation of certain acids and gases. Some of these characters may be occasional and not of vital importance to the continuance of the organisms. In the study of a group of oganisms which produce pigment, for example, the fluorescent types, many of the members of the group where this property of the production of coloring matter is at a minimum (or does not exist), may be missed on culture plates. One by one the marks by which different species are known have been wiped out by subsequent investigation until today the old species do not exist in many cases. Many of the marks by which their original investigators knew them have been found to be characteristic of unsuspected species. The so-called "urea bacilli" of Miquel, for example, seem to be only varieties of other organisms which have the ability to split up urea.[36]

When thermophilic organisms were first discovered, each new isolation, if it had a few other distinguishing characters, was made a new species, but recent studies in this group have shown that in nearly all cases the so-called "thermophilic species" are only strains of other organisms which have the peculiar property of living and growing

in extremely warm habitats; the result, undoubtedly, of selection and adaptation to this particular environment.[41,42]

RECENT ATTEMPTS AT DIFFERENTIATION

The skill of the bacteriologist has been exerted to find new and reliable tests which will differentiate between two nearly related organisms. Three characteristics of bacteria to which much recent attention has been given are: (1) reaction of various species to a combination of iodine with one of the anilin dyes, usually known as "Gram's stain," (2) acid-fast property by which some species hold anilin colors against decolorization with acid alcohol, (3) serological tests indicating affinities of proteins.

Staining Reactions as a Means of Differentiating Species of Bacteria.—No account of attempts at the classification of bacteria would be complete without a discussion of staining reactions.

Bacteria, killed by coagulation, take up and retain many of the anilin dyes; some species do this better than others, and some dyes are preferable to others. Not enough difference occurs, however, in the reaction of bacteria to ordinary anilin colors in aqueous solution to make them of any real diagnostic value in the determination of species.

Two combinations of anilin colors with mordants have been devised and are of real importance in separating species, as they reveal, by color reactions, something of the chemical or physical constitution of the bacterial cells. These combinations are:

1. Gram's anilin-gentian-violet stain.
2. Ziehl's acid-fast reaction.

Gram's Stain.—Gram's stain has become such a routine procedure in the determination of bacterial species that its diagnostic character and the theoretical basis for the reaction have been the object of much recent investigation.

The Stain.—Gram[18] noted that certain bacteria, such as those isolated from tubercular nodules, pneumonia, and in diseased tissue, when stained in an aqueous solution of gentian violet with the addition of a little anilin oil, would retain the color after washing successively with iodine solution (iodine, 1 part; potassium iodide, 2 parts; water, 300 parts) and absolute alcohol. The time for each of the three procedures was definite, 1 to 3 minutes in the stain, the same time in the iodine solution, and ½ minute in the absolute alcohol.

The bacteria which retain the color after this treatment were considered to be *Gram positive;* those which became colorless, *Gram negative.* Many of the pathogenic types are Gram positive, and for a time it seemed that perhaps pathogenicity and Gram positiveness were associated, but many purely saprophytic species (also the yeasts) are Gram positive, while some pathogenic ones are Gram negative.

The procedure is so valuable, however, that every organism is now described in its reaction to the Gram stain and numerous modifications of the original technic have been suggested.[6,29,30]

The dye usually used is a mixture of the pararosanilins, known under the Grübler trade name of "gentian violet," but other similar, slightly different, chemical combinations, produce nearly the same result. Anilin oil in aqueous solutions serves as a mordant, fastening the dye to the body of the organisms. Other bleaching reagents than absolute alcohol have been suggested, among them being various percentages of alcohol, clove oil, and acetone, but absolute alcohol has held its precedence in general use.

The results obtained, whatever the technic, are not always clear cut; at one time an organism retains the strain, while again it is decolorized. Such variations make its use of doubtful value as a diagnostic character and have led to investigations to solve the cause of such variability and, beyond that, to find the fundamental cause in the cells for the Gram reaction.

Variability in the reaction seems to be dependent on the age of the cultures. The majority of the Gram positive organisms stain more constantly from young cultures, but soil organisms react more strongly when the growth is 4 to 7 days old.[29] The only safe procedure, therefore, is to stain bacteria from cultures of three ages before pronouncing them to be Gram negative. With some organisms, the variability occurs rarely, with others, care and repeated trials are necessary before a decision can be made.

Gram positiveness is usually associated with an acid condition of the cell, a pH of 2 to 3, and an affinity for basic dyes of all sorts. The Gram negative bacteria have a pH centering around 5.[45,46]

In Gram-positive cultures, some organisms which are Gram negative can sometimes be isolated and propagated as variants or mutants.[44]

Attempts made to develop a Gram positive strain from a Gram negative organism, such as *B. subtilis*, by culturing them in solutions which contain a small percentage of gentian violet (1 part in 1,000,000 to 1 part in 6,000,000) have not been very successful. Gram-positive organisms seem to be more acid than the negative ones, and unite, for this reason, with readiness with a basic dye.[43] By selecting colonies of positive organisms which contain a maximum number of Gram negative mutants for growth on acid media, the percentage of negative organisms can be increased, but the strain can never be converted into a purely negative one, since the reaction of the bacterial body can only be changed slightly by growth on acid or alkaline media.

The theoretical explanations offered for Gram positiveness or negativeness are:

1. That it is a chemical reaction.
2. That it is a physical phenomenon.
3. That it is located in a special outer layer of the cell.
4. That it is associated with certain fat-proteins distributed throughout the cell.

These various view points cannot be entirely reconciled unless organisms differ in properties and mechanism for retaining the dye.[33]

The majority of the investigators of the Gram reaction hold that (1) some form of chemical affinity seems to exist between parts of the bacterial cell and the basic gentian violet, or (2) the gentian-violet-iodine molecules are too large to pass out of Gram-positive cells while they can escape from Gram-negative ones. The Gram-negative cells admit gentian violet in aqueous solution but allow it to pass out after the application of the iodine solution and alcohol. This condition means that this large molecule must pass through the outer membrane of the cell; as long as a Gram-positive bacterium has intact membranes its positive staining property is maintained, but if the membrane is destroyed or ruptured, the remains of the cell will be Gram negative. In this connection, the work of Schumacher[43] on yeast cells will be noted later.

The Location of the Colored Area.—The region of Gram positiveness is not yet definitely determined. Schumacher[43] is very pronounced in the opinion that the stain is held in lipoproteins forming a part of the interior of the cells and probably identical with the chromidial granules, generally considered to represent the bacterial nuclear material. On the other hand, Gutstein,[19–22] by a preliminary bath in a tannin mordant followed by basic stains, is able to differentiate in Gram-positive bacteria an outer layer which he terms "ectoplasm" which holds the stain. The ectoplasm is dual in nature, a basic ground substance with an acid impregnation, probably a lipoid.[21] The staining reaction is between the basic stain and the basic ground substance in acid dye solutions and between the lipoid and the acid dyes in basic solutions. The spore walls of Gram-positive bacteria also carry a basic ground substance bound up with an acid Gram-positive lipoid which reacts in the same manner as the ectoplasm composed of a Gram-negative lipoid bound to a basic ground substance.[20,21]

The ectoplasm, as understood by Gutstein, is the *membrane* of the bacterial cell and is not the outer layer of the cytoplasm. It is to be distinguished[19] also from the cell wall of organisms, such as *B. tuberculosis,* which contains a Gram-positive lipoid distinct from the ectoplasmic layer.

Gutstein does not deny, however, that the bodies of the Gram-positive bacteria still contain a lipoid which can be stained by basic dyes even after the organisms have been made artifically Gram negative by chemical treatment, this lipoid (as granules) being probably the nuclear substance. It will not retain the dye inside the cell, however, and bleaches in alcohol.

Gutstein and Schumacher are in agreement as to the chemical nature of the chromidial granules, but Schumacher denies the importance in Gram staining of the ectoplasmic layer.[43] The staining of the chromidial granules, when bacteria are grown on solid media in which dyes have been dissolved, has been known for a long time,[47] but Schumacher has extended this absorption of coloring matter to include the Gram stain and has based on it his theory of disinfectants (p. 422).

The ordinary yeast plant is Gram positive. Schumacher[43] very ingeniously tried to determine whether the positiveness rested in the outer layer or in the cell contents, the yeast plant being chosen for this purpose probably because of its size.

Yeast cells frozen and cut on a microtome into 5-μ sections exposed the contents of many of them to the direct action of the stain without any passage through the ectoplasm. The contents were Gram positive while the empty cell membranes were Gram negative, indicating that the intactness of the outer layer is not necessary for Gram positiveness in the yeast plant.

Kalina[34] describes the Gram reaction as consisting of two phases: (1) a physical adsorption of the dye by the ectoplast; (2) a chemical reaction of dye, iodine, and lipoid to form internally a compound insoluble in alcohol. The chemical reaction would be the important one, so that his viewpoint is similar to that of Schumacher.

The chemical basis for Gram positiveness rests in a lipoid impregnating the outer cell membrane (Gutstein) or the chromidial granules (Schumacher). The location of the Gram-positive portion of the cell is still a matter of dispute between these two investigators but it may not be the same in all organisms.

Acid Fastness in Bacteria.—The acid-fast property in bacteria is distinct from the Gram reaction, but is due to the presence of similar fatty and lipoid compounds, as already noted under the tuberculosis organism (p. 283). A number of other bacteria retain a stain against the action of weak acids: *B. leprœ*, *B. smegmatis*, and the so-called "timothy organisms." The presence of lipins has been demonstrated in all of them, but their location has not been exactly determined, although a large part is in the cell wall. The extraction of the fatty compounds from the tuberculosis bacillus by the use of light oils, such as xylol, will not render an acid-fast organism non-acid fast, so that some other substance must be partly responsible for the tenacity with which the cells retain the dye[50]. On the other hand, the substance which is removed by such treatment is itself acid-fast. The cells retain enough of this compound, even after extraction with xylol, to render their reaction positive, this last trace, which has not been removed and which gives the reaction, apparently being held as a lipin by the proteins in some combination from which ordinary fat solvents will not remove it.

The two staining reactions just described are always on the lists of the essential descriptions of an organism, and, in spite of their variation and frequent uncertainties, are valuable aids in distinguishing between related varieties.

The Separation of Varieties of Bacteria by Serological Methods.—Serological methods supposedly give some clue to the comparative protein constitution of the bacterial cells.

The success of this method in determining the typhoid and related organisms (p. 265) has led to its use in other groups. The lack of such clumping phenomena, even with bacteria that are very similar in morphological and cultural characters, places a new instrument into the hands of the student of closely related species for their separation.

Agglutination is placed[4,5] among the phenomena associated with colloids, explainable by the principles of chemistry, and not a special life process. It may be due to an internal rearrangement of atoms in the molecule of the bacteria, this reconstruction being induced by some chemical, present in the agglutinating fluid, or by the action of heat, water, or other physical agent. Or, the bacterial protoplasm may absorb certain ions from the agglutinating medium which change their electrical charge, increasing the *attracting* forces and lessening the *repelling* ones, with the result that the

bacteria clump, it being assumed that the ordinary condition of organisms is one of repulsion to each other.

The pathogenic organisms have been studied with care to determine the matter of agglutination, sometimes with good results, and at other times without much addition to the physiological and cultural data already known.

Non-pathogenic organisms recently have been subjected to the same tests. One of the earlier of these applications, made by Hefferan[26] on the *B. prodigiosus* group, showed marked differences in some of them. One of the very puzzling groups of bacteria is that of the root tubercles of the legumes where the number of physiological species is still a matter of dispute; nearly all the data are derived from cross-inoculation experiments with the different strains of organisms isolated from the various legumes upon other legumes. Vogel and Zipfel[43] prepared immune sera of strains of the legume bacteria by growing them on culture media, removing the organisms and inoculating them into rabbits. The blood of the rabbit produces antibodies against a particular organism or any closely related species with the same type of protein. If into this serum (diluted 1–5,000 or 1–10,000) of the rabbit's blood were then placed cultures derived from the same legume or strains from other legumes, the individual organisms would be affected. If a culture of the nodule bacillus from the same plant is added to the serum derived from the same strain of organism, the bacteria will agglutinate, indicating that they are affected, superficially, by some substance deposited in the serum from the stimulus of the organism. For example, the immune serum derived from an inoculation of *Trifolium* organisms will cause other organisms derived from the same plants to clump, as it will, also, the bacteria from cultures isolated from other varieties of clover, but would not agglutinate those from peas, beans, lupine, or medicago. The same general results were obtained within the *B. radicicola* group as had been obtained by the cross-inoculation experiments, five main strains being represented. Fred, Whiting, and Hastings[17] inoculated the strains into rabbits and used rabbit serum for the agglutination tests, dividing *B. radicicola* into six agglutination groups.

Hucker[28] separated 15 species of *Micrococcus* by the use of the serological methods but doubts if larger groups can be differentiated in this way. Apparently, different species are sufficiently unlike to produce this reaction but very little can be learned about larger groupings from this reaction to serological tests.

The serological method undoubtedly has possibilities, and will undoubtedly be used more and more for the determination of species[23] since, as has been shown, the older criteria are so doubtful and variable. The most permanent character about an organism, large or small, should be its proteins, and, by serological methods, proteins can be compared.

Nomenclature as a Practical Problem.—Nomenclature and classification will be recognized as being two distinctly different problems. Nomenclature is the practical problem of finding a name for an organism which will carry a fairly definite meaning to other students of the bacteria. Classification should represent the real relationships of the organisms. The two should be the same, but if a real classification is impossible at the present time, nomenclature is not.

"Bacteriology is a science which from the beginning has had its practical and economic applications. A larger proportion of the known species

of bacteria are of economic importance probably than in any other plant group. Uniformity and stability in nomenclature are therefore even more desirable in this group than in most others; the scientific names are more widely used by those not particularily interested in problems of taxonomy."[4a]

The conservative viewpoint with regard to nomenclature is represented by Hall[25] who makes a plea "for definitions of bacterial genera and all larger groupings upon exclusively morphologic (including tinctorial) criteria, reserving for the definitions of species physiologic properties, and excluding serological reactions, particularly agglutination." A similar attitude is assumed by another recent writer on classification, Janke,[31] who still believes that the real relationships of the bacteria are indicated by morphological, rather than physiological characters, and adheres to seven generic names: *Streptococcus*, *Micrococcus*, *Sarcina*, *Bacterium*, *Bacillus*, *Microspira*, and *Spirillum*.

On the other hand, the physiological and chemical advocates hold that the chemical constitution of the proteins of the bacteria are more stable and fundamental than any structural differentiation.[40]

The older morphological groups will probably remain as the basis of nomenclature for some time at least, with species separated by the physiological and possibly their serological reactions. This method will serve the purposes of nomenclature, and, as little is really known as to relationships, must answer until more light is thrown on bacterial classification.

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